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14. ABSTRACT

Genetic susceptibilities for breast cancer can be elucidated by studying genotype-phenotype correlates. This study investigated women from high risk breast cancer families, a sporadic breast cancer case-control study and associations for specific *BRCA1* SNPs and haplotypes, *Rad51* SNPs, and deficient DNA repair. The mutagen sensitivity assay (MSA), used to measure DNA repair capacity, was used to test associations with genotypes and haplotypes. Positive associations were then tested as predictors of breast cancer risk in a population-based case control study. *BRCA1* carriers with breast cancer had more mean breaks per cell (MBPC) than *BRCA1* carriers without breast cancer. An association was found for the *Rad51 5'UTR 135C* allele and MBPC (OR=3.40 95% CI: 1.20-9.90). There also was an increased risk for high MBPC with the *BRCA1 D693N* allele (OR= 6.03 95%CI: 0.69-52.02; p=0.10). There was no association with for the *BRCA1 Q356R* and *E1038G* genotypes or haplotypes. The *Rad51 5'UTR 135C* allele was examined in a population-based case-control study of breast cancer, but no association was found. The results indicated that the *Rad51 5'UTR 135C* allele and maybe the *D693N* allele are modifying genotypes for the penetrance of *BRCA1* mutation carriers, and so might only be risk factor for high risk families.

15. SUBJECT TERMS
DNA repair, radiation sensitivity, BRCA1, Rad51 polymorphisms, hereditary breast cancer, sporadic breast cancer, Rad51, SNPs, halotypes, genotypes

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Introduction and Significance

Breast cancer is the most common cancer among women, except for non-melanotic skin cancer, and the second leading cause of cancer death in women living in the United States (ACS 2007; www.cancer.org) (1). In the U.S., a woman's lifetime risk for developing breast cancer is 13% (1 in 8). In 2006, more than 200,000 new cases of invasive breast cancer were diagnosed, making North America's rate the highest in the world.

The genetic determinants of why some women develop breast cancer and others do not is under intensive study. A subset of women who have a strong family history of breast cancer inherit *BRCA1* and *BRCA2* mutations, which have a very high penetrance. However, penetrance varies from 46 to 85% (2-6), and so the factors that contribute to cancer risk in these women are unclear, although it has been reported that a family history of breast cancer and reproductive factors might be additional risks factors affecting penetrance in high risk families (7-16). Ultimately, factors influencing penetrance of high risk gene mutations might be gene-environment interactions for *BRCA1* or other high risk genes, gene-environment interactions of other genes that are less prevalent, or gene-gene interactions of genes that interact with the high risk genes. For sporadic cancer risk, many low penetrant traits have been investigated in pathways ranging from estrogen metabolism to DNA repair, but it has been difficult to find consistency across studies.

There are genes and genetic polymorphisms that may modulate hereditary and sporadic breast cancer risk, but little guidance on which ones to study. Many polymorphisms have been identified, but the relationship between the allelic variant and its possible alteration in protein function is usually unknown, and for many polymorphisms, *a priori* hypotheses are difficult to

establish. The likelihood of finding false-positive associations is greater than true-positives unless there are specific *a priori* hypotheses (17, 18). That is why a candidate-gene and candidate-pathway approach was used in this study. This pathway-based genotyping tactic will examine the combined effects of several polymorphisms that act together in the same pathway and this way, we increase the likelihood of a true positive-correlation between a genotype and phenotype. BRCA1 and *Rad51* were chosen to be examined because of their involvement in the HRR DNA repair pathway. In theory, they could ultimately affect breast cancer risk, especially in already DNA repair compromised *BRCA1* mutation carriers. Because of BRCA1 and *Rad51*'s function, we propose that polymorphisms in both genes could consequently lead to deficient DNA repair, discoverable by studying women whose DNA repair may already be compromised. In this study we will determine if there is a relationship between genetic polymorphisms for BRCA1 and *Rad51*, and the haplotypes with the MSA (mutagen sensitivity assay) phenotype. Then, results will be used to identify genotypes associated with breast cancer risk in a population-based case-control study.

There are different ways that genotypic traits can be assessed for cancer risk. These include the studying of genotypes and single nucleotide polymorphisms (SNPs), haplotypes, and phenotypes. A SNP is similar to a mutation in that it encompasses DNA sequence variation occurring when a single nucleotide in the genome differs between members of a species. The main difference is in its frequency; SNPs have allele frequencies of $\geq 1\%$ whereas mutations are much less. It is important to note that SNP frequencies can vary substantially by ethnicity and race. SNPs may fall within coding, non-coding, or in the intergenic regions between genes. However, SNPs within a coding sequence will not necessarily change the amino acid sequence

of the protein that is produced because of redundancy in the genetic code, and so are called *synonymous* SNPS. If the change in a base at a SNP results in a different polypeptide sequence, it is called *non-synonymous*. SNPs that are not in protein coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA. SNPs make up 90% of all human genetic variations, and SNPs with a minor allele frequency of $\geq$1% occur every 100 to 300 bases along the human genome (19).

A haplotype or haplotype block is a set of single nucleotide polymorphisms (SNPs) on a single chromatid that are inherited jointly through evolution. Thus, studying some SNPs within a block can represent inheritance patterns for other SNPs within the block (19). This is called linkage disequilibrium and it is the non-random association of two or more alleles at different loci. Non-random associations between these alleles are measured by the degree of linkage disequilibrium, D'. Haplotype-based association studies have been proposed as a comprehensive approach to identify causal genetic variation underlying complex diseases.

Candidate gene studies, whether focusing on SNPs or haplotypes, pose challenges because the effect of a single gene variant within a pathway might not be sufficiently penetrant to show a change in risk. Even when results show small functional changes in protein structure or activity or demonstrate low allelic frequencies in the population, there are still problems with statistical power. However, phenotypes are generally regarded as complex genotypes and have the capacity to show the predictive value of single genetic traits using intermediate indicators of carcinogenesis. A phenotype is the observable physical or biochemical characteristics of an organism, as determined by both genetic makeup and environmental influences. Therefore, phenotype penetrance could be higher and more apparent in small association studies.

The phenotype measured in this study will be DNA repair capacity and it will be measured by the mutagen sensitivity assay (MSA). The MSA (mutagen sensitivity assay), also known as the radiation-induced G2 chromatid break assay, has been proposed as a phenotypic assay of breast cancer risk for both familial and sporadic breast cancer related to DNA repair deficiency (20-27). This assay measures the number of chromosomal breaks following exposure of cultured lymphocytes to DNA damaging agents. Gamma radiation has been the most widely used mutagen in breast cancer studies because it is a direct DNA damaging agent that is not dependent of cell penetration, metabolism, or clearance. There is much evidence to support the hypothesis that the MSA (mutagen sensitivity assay) is a good biomarker for DNA repair capacity in the HRR pathway (28-32). By using the phenotype of DNA repair capacity, valuable data set of EBV-immortalized lymphoblasts from subjects with *BRCA1* mutations and their family members were utilized to investigate the association between genotype and DNA repair phenotypes. These cell lines, and also fresh blood, were provided by the Lombardi Comprehensive Cancer Center Familial Cancer Registry.

In the first part of the study, the mutagen sensitivity assay (MSA) was established and validated as a marker of DNA repair capacity. Then, this assay was applied to cell lines from 138 women and the range or MBPC (mean breaks per cell) were established. Sequence data for BRCA1, determined by Myriad Genetics, and data provided by sequencing of *Rad51* was used to identify SNPs and haplotypes associated with MBPC (mean breaks per cell). Associations found were then tested in an existing population-based case-control breast cancer study, funded by the NIH and DOD. This case-control study breast cancer from the University of Buffalo studies the relationship between alcohol, diet, oxidative damage and sporadic breast cancer.

This study will differ because the use a phenotypic, functional assay to assess DNA repair will determine genotype-phenotype associations which will identify functional variants that could be associated with breast cancer risk. This study was designed to provide a model for the measurement of functional consequences of genetic variation and to identify new genotype-phenotype relationships. This allowed for the identification of potential modifying genes that would affect the penetrance of *BRCA1* mutations and allowed for prioritization of genes and SNPs to assess in future genetic epidemiological studies of breast cancer risk.

The significance of this study lies in discovering how polymorphisms in DNA repair genes affect *BRCA1* penetrance and observable phenotypes and how this information could be applied to sporadic breast cancer. The purpose, herein, was to explore possible mechanisms for increased breast cancer susceptibility for DNA repair capacity using genotype-phenotype methods and to identify genetic variants associated with phenotypic biomarkers and breast cancer risk. This study used an *in vitro* model for DNA repair in order to establish *a priori* hypotheses for functional effects of genetic variants that might affect both high risk family and sporadic breast cancer risk, and test them in a population-based case control study.

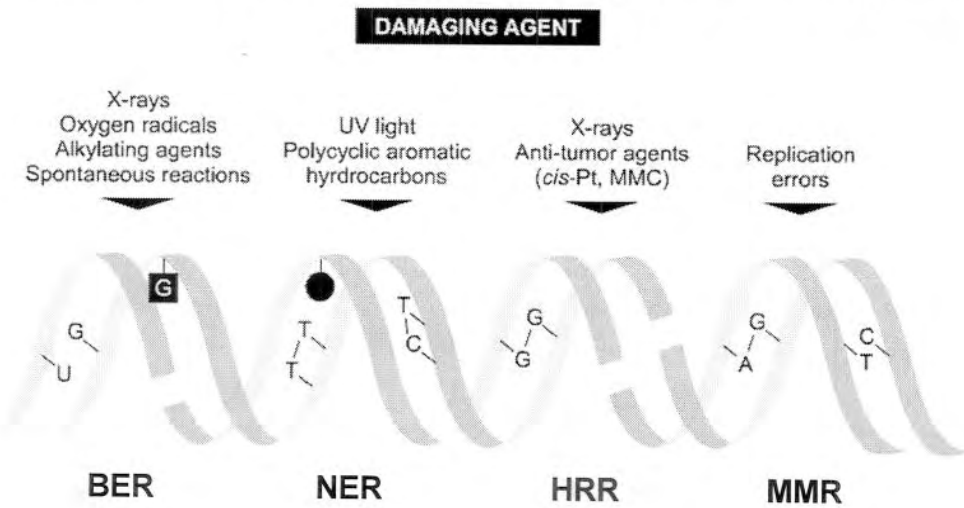
For this study, the plan was to utilize several unique resources and capitalize on the high risk group of women to provide important information about high-risk and sporadic breast cancer. Ultimately, the candidate-gene/ candidate-pathway approach was used in this genetic association study to test hypothesis about a causal pathway. These studies could aid in the development of more rationale prevention strategies by increasing the accuracy and successful assessment or prediction of breast cancer risk.

DNA Repair Pathways

Maintenance of genomic integrity is critical for the survival of all living organisms. DNA is under continuous threat from endogenous and exogenous genotoxic agents. Following genotoxicity, there are complex and redundant DNA repair mechanisms, and when the DNA cannot be repaired, there is a trigger for apoptosis or it can trigger progression through the carcinogenic process (33, 34).

At least four DNA repair pathways are known to operate on specific types of damaged DNA. Each pathway involves numerous, but different molecules and are illustrated in Figure 1, which was adaptation from Hoeijmakers et al (33). Three of the DNA repair pathways, namely base excision repair, nucleotide excision repair, and mismatch repair carry out single-stranded break repair. Double stranded break repair is carried out by homologous recombinational repair (HRR) and non-homologous end joining to repair (NHEJ).

Figure 1. DNA damage, repair mechanisms, and consequences. Common DNA damaging agents (top); examples of DNA lesions (middle); and most relevant DNA repair mechanism responsible for the removal of lesions (bottom). Adapted from Hoeijmakers et al (33)



Base Excision Repair (BER), Nucleotide Excision Repair (NER), and Mismatch Repair (MMR)

Base-excision repair (BER) (Figure 2a) operates on small lesions such as fragmented or non-bulky adducts, or those produced by methylating agents. BER also defends against damage caused by oxygen radicals, spontaneous deamination, and hydrolysis. It is mostly responsible for recognizing minor damage to bases and the DNA sugar backbone. The molecules involved in BER often recognize one substrate, remove the damage, and leaves the sugar phosphate backbone (33, 35). The main BER pathway repairs SSBs (single stranded breaks) formed by enzymatic cleavage, gamma rays, or chemicals like bleomycin, H_2O_2, nitrogen oxide (NO), and etoposide. XRCC1 has a central role in the main pathway. It has no enzymatic activity by itself, but rather functions as a loading factor for polymerase beta and ligase III (36). BER is active in the G1 phase of cell cycle, but also in the G2 phase (37).

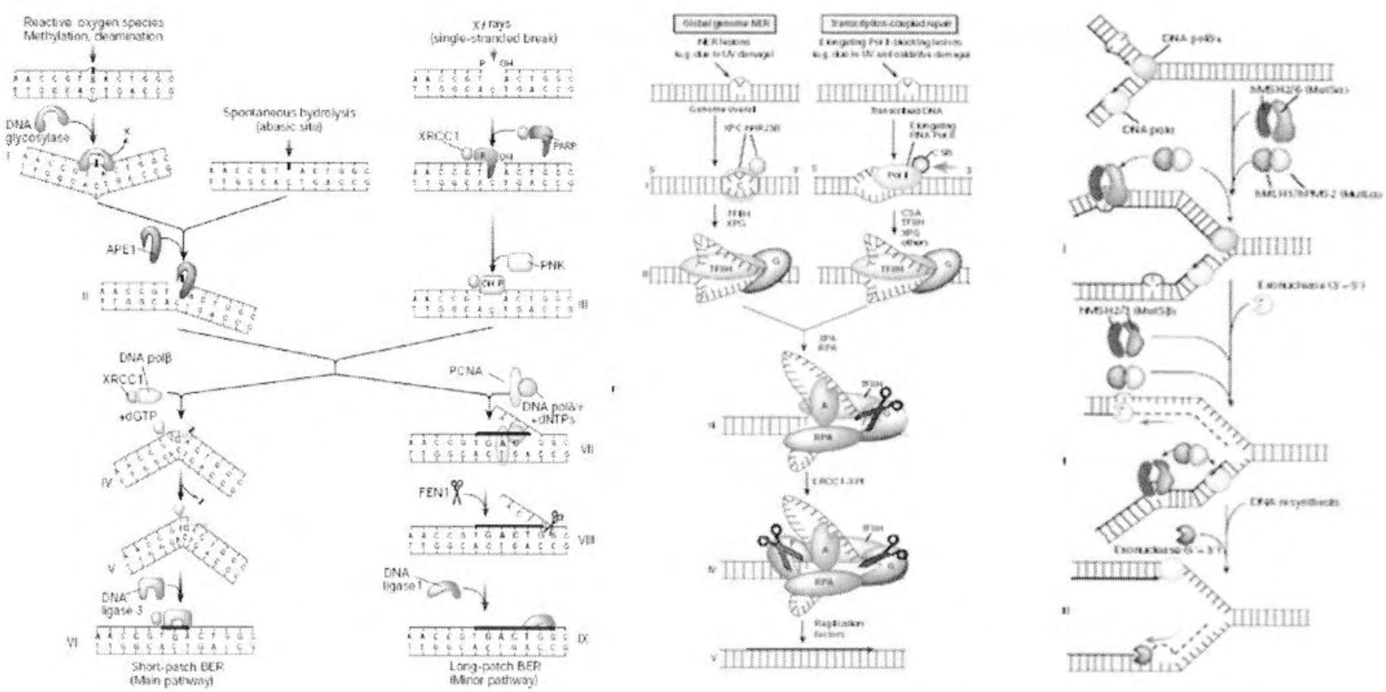
Unlike BER, NER (nucleotide excision repair) is responsible for repairing a large number of different bulky DNA lesions, such as pyrimidine dimers, photo-products, larger chemical adducts, and DNA intra-strand cross-links. For example, NER is responsible for the removal of benzo[*a*]pyrene (B[*a*]P) adducts, which distort the conformation of the DNA helix. NER is so far the only known mechanism in the human body that removes the major UV-induced photo lesions (38). Also, small lesions caused by reactive oxygen species (ROS) can possibly be repaired by NER (39).

There are many proteins involved in NER and the separate steps can be divided into DNA damage recognition, DNA unwinding, 3'- and 5'-incision, patch repair synthesis, and ligation. The main proteins include XPA and RPA (replication protein A, damage recognition),

XPB and XPD (DNA unwinding helicase and ATPase), XPC (DNA binding), XPF (5' nuclease), XPG (3' nuclease), and ligase I (nick sealing) (40-43). Disorders linked to deficient NER include Cockayne's Syndrome and Xeroderma Pigmentosum, diseases associated with a high risk for UV-induced cancer (33).

Another category of DNA repair is mismatch repair (MMR), which corrects replications errors caused by DNA polymerase (44). Hereditary non-polyposis colorectal cancer (HNPCC) is a consequence to dysfunctional MMR. It involves mutations in *MSH2* and *MLH1,* where these mutations are involved in almost half of all cases of HNPCC (33). Figures for the aforementioned DNA repair pathways from Hoeijmakers et al are used here to illustrate the different pathways (33).

Figure 2. a) BER, b) NER , and c) MMR Pathways (33)



Double Stranded Break Repair in Eukaryotes

Human cells can also process double-stranded DNA breaks (DSBs), which involve the actual breaking of both sugar-phosphate backbones and complete separation of DNA from chromosome. Double-stranded breaks (DSB) can be produced by replication errors, from endogenously generated reactive oxygen species and free radicals that are produced during normal cellular metabolic reactions and by exogenous agents such as ionizing radiation or certain chemotherapeutic drugs, such as cisplatin and mitomycin C (33, 34, 45); they can also be produced when DNA replication forks encounter DNA single-strand breaks (46). In addition, eukaryotic cells naturally produce DSBs during meiosis and VDJ recombination. Moreover, inaccurate rejoining of broken ends can occur and this can result in loss or amplification of chromosomal material or to translocations where segments of a chromosome are exchanged (46), resulting in apoptosis or tumorigenesis. To compensate for different types of damage, two different pathways involved in double-strand break repair have evolved: homologous recombinational repair (HRR) and non-homologous end joining (NHEJ).

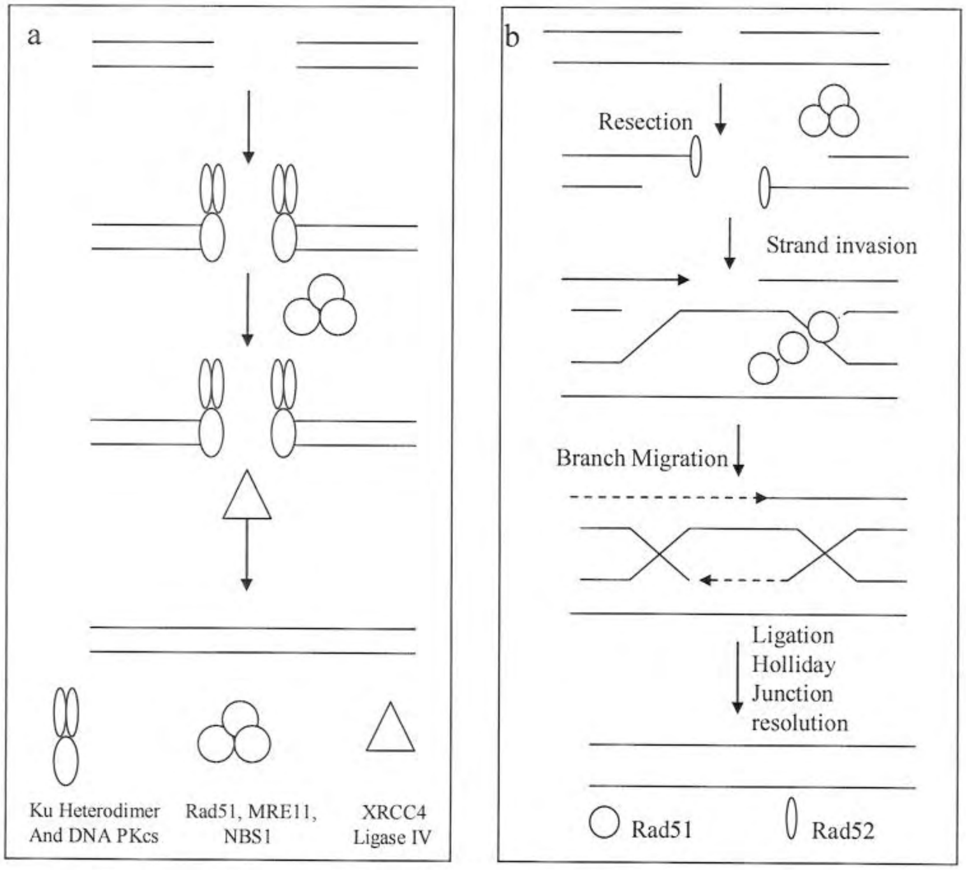
These two pathways differ in their requirement for a homologous DNA template and the fidelity of the repair reaction. Figure 3 is an illustration of each pathway and was adapted from Khanna et al (46). HRR uses an intact sister duplex as a template for repair, so the reaction occurs with high fidelity, whereas NHEJ occurs without a template and involves direct ligation of the broken ends. Also, while NHEJ occurs mostly in G1 phase, HRR occurs in S and G2 phases, making these pathways, cell cycle dependent (47-49).

Non Homologous End Joining (NHEJ)

Non-homologous end joining repair (Figure 3) involves the direct ligation of the two double-strand-breaks and generally leads to small DNA sequence additions or deletions. DNA ligase IV directly mediates the DNA strand-joining events and functions in a complex with XRCC4 (50). The ligase IV/XRCC4 complex does not recognize the site of the break, but is recruited by DNA-dependent protein kinases. The DNA-end-binding protein Ku, binds free DNA ends and recruits the DNA-PKCs. XRCC4 and ligase IV are recruited directly or indirectly by the DNA-PK holoenzyme and are activated by DNA-PK–mediated phosphorylation. The Rad50-Mre11-Nbs1 complex, which contains helicase and exonuclease activities, may also function in NHEJ, particularly if the DNA ends require processing before ligation.

Although HRR is more accurate, cells do not always have a homologous sister chromatid available and NHEJ does not require an undamaged partner molecule so it does not rely on extensive homologies between the second DNA duplex and the two recombining ends. Additionally, it does not require reshuffling of other proteins bound to DNA as HRR does. Therefore, NHEJ is rarely error-free.

Figure 3. Pathways involved in repair of double-stranded break repair. A) Non-homologous end joining B) homologous recombinational repair (46).



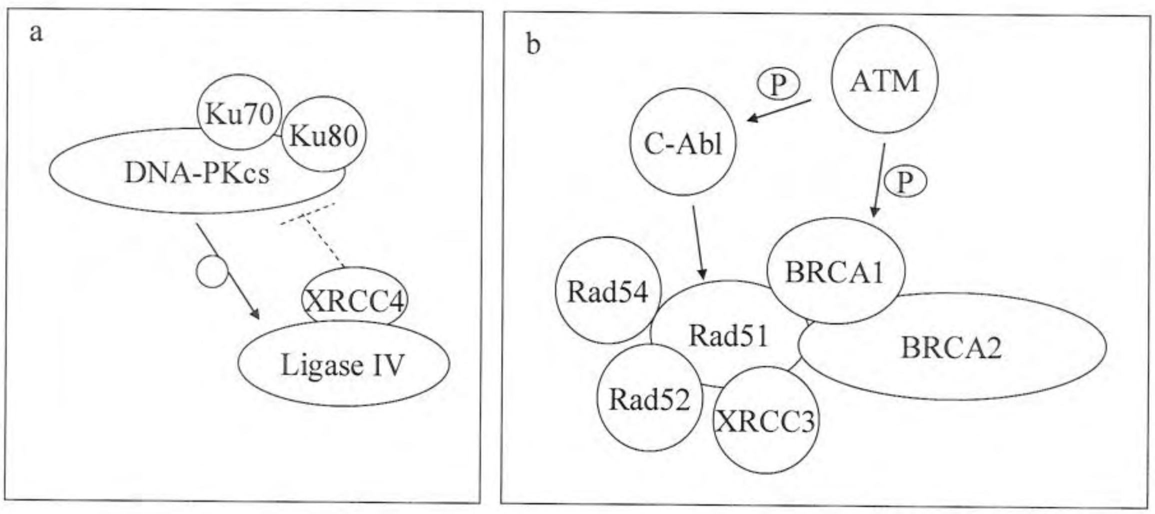
Homologous Recombinational Repair

Homologous recombinational repair (HRR) (Figure 3) is the second pathway involved in the repair of DSBs. Briefly, the DNA ends of the break are resected in the 5' to 3' direction by nucleases; the resulting 3' single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule, and are extended by the action of DNA polymerase, which copies information from the partner.

HRR requires proteins such as Rad52, a DNA-end-binding protein, and *Rad51*, which forms filaments along the unwound DNA strand, to facilitate the strand invasion. Specifically,

the strand-exchange reaction catalyzed by *Rad51* is facilitated by Rad52 through direct interaction. Rad54, a DNA-dependent ATPase, also interacts directly with *Rad51* and stimulates its activity. *Rad51*-related proteins (*Rad51*B-D, XRCC2 and XRCC3) are also involved in HRR. *Rad51* also interacts with BRCA1 and BRCA2 and they colocalize to the site of the break when damage is encountered. The proteins involved in NHEJ and HRR DNA repair pathways are illustrated in figure 4 (adapted from Khanna et al) (46). and protein functions involved in HRR are listed in Table 1.

Figure 4. Double stranded break repair pathways. a) Non-homologous end-joining b) homologous recombinational repair.



Proteins involved in Homologous Recombinational Repair

There are many components to the double-stranded break repair pathways (Table 1). At present, we will focus of the HRR pathway because 1) it is the pathway induced by ionizing radiation and 2) it involves BRCA1 in the DNA damage response pathway, which is one of the genes of interest in this project.

Table 1. Function of genes involved in homologous recombinational repair.

Gene	Function
ATM	Nuclear protein kinase (Ser/Thr); phosphorylates and activates BRCA1
ATR	Nuclear protein kinase (Ser/Thr); phosphorylates and activates BRCA1
BLM	DNA helicase, interacts with BRCA1
BRCA1	Nuclear Protein; Recruits repair proteins to DNA damage site;interacts with BRCA2 and Rad51
BRCA2	Nuclear protein; Recruits repair proteins to DNA damage site ;interacts with Rad51
c-Abl	Non-receptor tyrosine kinase
MRE11	nuclease that interacts with Rad51 and NBS1 required to initiate DSB repair
NBS1	Enhances nuclease activity of MRE11
P53	transcriptionally regulates genes involved in DNA repair
Rad50	Enhances nuclease activity of MRE11
Rad51	Binds ss and promotes homologous pairing and SCE
Rad52	Interacts and enhances activity of Rad51 in HR
Rad54	ATP-dependent ATPase; stimulates Rad51 pairing stabilizing complex and SCE
Rad54B	Interacts and enhances activity of Rad51 in HR
XRCC2	Interacts and enhances activity of Rad51 in HR
XRCC3	Interacts and enhances activity of Rad51 in HR
WRN	Helicase which unwinds DNA

BRCA1

BRCA1 is a nuclear protein found on chromosome 17q21 and it contains 24 exons that encode an 1863 amino acid protein (genomic accession no: NM_007306). It was isolated by Miki et al in 1994 (51) and sequence analysis reveals that the N-terminus contains a zinc RING finger domain found to be involved in protein to protein interactions (52). Specifically, the N-terminus interacts with other DNA repair proteins such as BARD, BAP, E2F and DNA. The C-terminus has two 95 base-pair residue BRCT (**BR**CA1 **C**-**t**erm) domains involved in DNA repair and the cell cycle (53, 54). The most C-term BRCT repeat is important for hetero-dimerization. *BRCA1* contains 2 nuclear localization signals (NLS) and contains nearby regions that interact with p53 and *Rad51*. Mutations in this region (exon 11) will leave BRCA1 dysfunctional (55), because the protein cannot localize to the nucleus. The C-terminal domain has also been shown to contain transactivation activity (56), and because of this, BRCA1 is believed to function as a

transcription factor. It has been shown that BRCA1 has a role in sensing DNA damage and checkpoint control of the cell cycle, and sensing DNA breaks triggers homology-directed DNA repair (57-63). BRCA1 deficient cells that have DNA repair defects are partially rescued by providing exogenous wild-type BRCA1 (60, 64).

Evidence is mounting that BRCA1 has local activities at double-stranded break (DSB) sites. Specifically, BRCA1 has a role in sensing DNA damage and checkpoint control of the cell cycle and it has been suggested that BRCA1's role in sensing breaks actually controls homology-directed DNA repair (60). In particular, BRCA1 is rapidly phosphorylated by the ATM and CHK2 kinases after DNA damage in dividing cells, suggesting that it may work downstream of checkpoints that sense and signal DNA damage or problems with DNA replication during S phase (57, 59, 63). After phosphorylation, BRCA1 migrates to the site of the DSB in a p53-dependent manner (65), where it co-localizes with *Rad51* in S-phase nuclear foci (66). Then MRE11/RAD50/NBS1complex is recruited (67, 68) where the exonucleolytic activity of the complex is mediated by MRE11 and is responsible for resecting DSB ends to generate single stranded DNA tracts (69). Recent work suggests that BRCA1 regulates the activity of this complex and under certain *in vitro* conditions, where BRCA1 can inhibit the activity of MRE11 (60), regulating the length and the persistence of single stranded DNA generation at sites of DNA breakage.

BRCA1's role in DNA repair was also verified in a functional study of a human breast cancer cell line (HCC1937) that contains a single mutant 5382insC BRCA1 allele; it was revealed that these BRCA1 deficient cells have a defect in repair of oxidative DNA damage caused by ionizing radiation compared to normal cells that do not contain the mutation (58, 70).

Other studies have found that BRCA1 deficient cells have DNA repair defects, which are partially rescued by providing exogenous wild-type BRCA1 (60, 64).

The structure of BRCA1 reveals several domains that interact with a variety of proteins that function in the ubiquination pathway, as well as the DNA repair pathway. The BRCA1 protein contains a RING finger domain (71) that results in homodimerization or heterodimerization with other proteins such as BARD1 (BRCA1-associated RING domain protein), which contains ubiquitin ligase activity (72-74). Their interaction is mediated by the flanking regions of the RING finger motif (75). These findings suggest that BRCA1 may have a direct role in DNA repair that is mediated by ubiquitin ligase activity.

Because of BRCA1's role in DNA repair, this project sought to determine how this aspect affects breast cancer risk. In general, women with mutations in *BRCA1* have a 46-85% chance of developing breast cancer in their lifetime (2-6). Hundreds of different *BRCA1* mutations have been reported in the Breast Cancer Information Core (BIC; www.nchgr.gov/intramural_research/lab-transfer/Bic) database and questions about the function of each mutation have arisen.

To understand how phenotypic differences arise, the functional domains in BRCA1 must be considered. The 185delAG mutation is found in exon 2, where the RING finger domain is found and because of its truncating effects, could affect protein-protein interactions in both DNA repair and ubiquination. The 5382insC mutation is found in exon 20 and also causes a frame-shift, resulting in a premature stop codon in the last exon of the gene. Because of this, it has been anticipated that this mutation could affect the regulation of transcription and could lead to decreased expression of BRCA1 protein (76, 77). Remarkably, the functional domains

suggesting a role in DNA repair are found around exon 11, and most SNPs found in *BRCA1* are found near this exon. Exon 11 encompasses the interaction domains with *Rad51* (61). Additionally, several residues phosphorylated upon different types of DNA damage by Chk2 (59), ATR (63), and ATM (57) lie within exon 11.

Many BRCA1 polymorphisms with allele frequencies >5% in Caucasians have been identified, and of the 32 BRCA1 polymorphisms listed on the Cancer Genome Anatomy Project SNP500Cancer Database (http://snp500cancer.nci.nih.gov/), 24 are found in coding regions. Of the 24, 18 are non-synonymous, but only 12 of the 18 have reported actual functional changes that go along with the amino acid changes (Table 2). These polymorphisms, with the exception of *Q356R* and *D693N*, are in significant linkage disequilibrium and generally are inherited as part of a shared haplotype (International HapMap project, www.HapMap.org).

Table 2. BRCA1 table of SNPs found in SNP500Cancer Database.

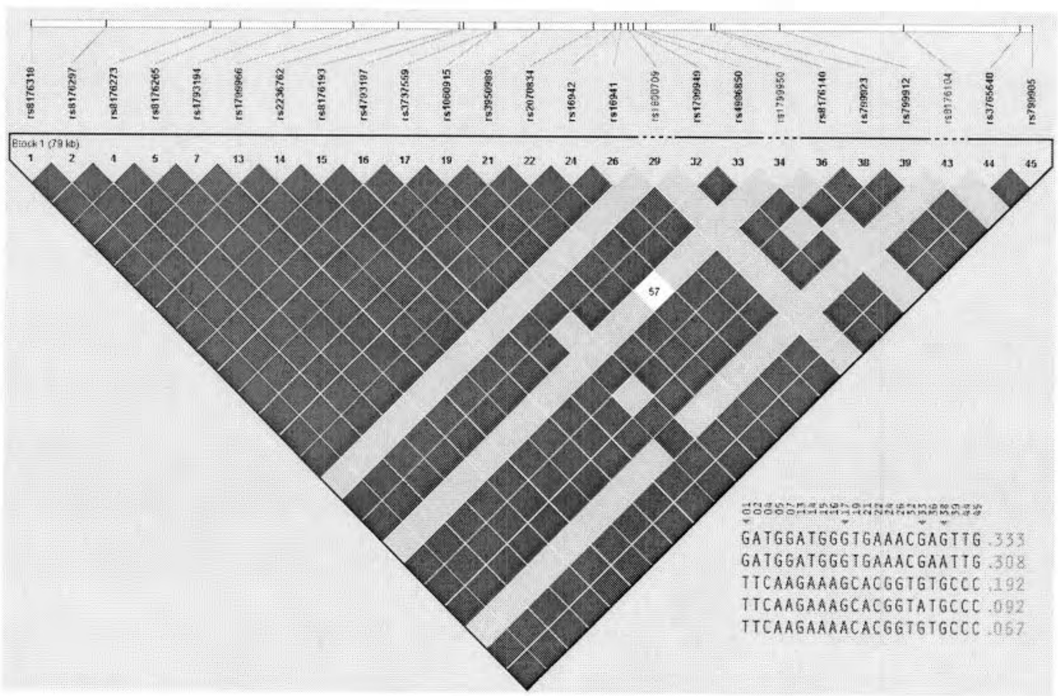
refSNP ID	SNP region	Amino Acid Change	Amino Acid Substitution	Functional change
rs4986850	Ex12+1407G>A	D693N	Aspartic Acid - Asparagine	acid - neutral
rs16941	Ex12-984A>G	E1038G	Glutamic Acid - Glycine	acid polar - neutral non-polar
rs4986848	Ex12-1131T>C	F948S	Phenylalanine - Serine	nonpolar - polar
rs1800707	Ex14+33G	K1406N	Lysine - Asparagine	basic - neutral
rs4986854	Ex17-104T>C	M1587T	Methionine - Threonine	nonpolar - polar
rs4986845	Ex12+1497A>G	N682D	Asparagine - Aspartic Acid	neutral - basic
rs1799950	Ex12+397A>G	Q356R	Glutamine - Arginine	neutral - basic
rs4986849	Ex14-30G>A	R1402Q	Arginine - Glutamine	basic - neutral
rs1800709	Ex12-1576C>T	R800W	Arginine - Tryptophan	basic - neutral
rs2227945	Ex12-679A>G	S1099G	Serine - Glycine	polar - nonpolar
rs1799966	Ex17-150A>G	S1572G	Serine - Glycine	polar - nonpolar
rs4986852	Ex12-978G>A	S999N	Serine - Asparagine	polar - nonpolar
rs4986847	Ex12-1324A>C	I884L	Isoleucine - Leucine	no change
rs16942	Ex12-549A>G	K1142R	Lysine - Arginine	no change
rs1799967	Ex17-31G>A	M1611I	Methionine - Isoleucine	no change
rs1800704	Ex12-1073G>A	M967I	Methionine - Isoleucine	no change
rs799917	Ex12-1485C>T	P830L	Proline - Leucine	no change
rs4986852	Ex12-978G>A	S1040N	Serine - Asparagine	no change
rs4986846	Ex12+1562T>C	A703A	Alanine - Alanine	no change
rs1800740	Ex12-1364A>G	G870G	Glycine - Glycine	no change
rs16940	Ex12+1641T>C	L730L	Leucine - Leucine	no change
rs1060915	Ex14-50T>C	S1395S	Serine - Serine	no change
rs1799949	Ex12+1412C>T	S653S	Setine - Setine	no change
rs4986844	Ex12+1439A>G	T662T	Threonine - Threonine	no change

BRCA1 Haplotyping

Haplotype analysis, particularly the use of tag SNPs, may prove useful for identifying genetic heterogeneity when functional alleles are unknown. In the case of BRCA1, it was found that most polymorphisms are in high LD and only a few tag SNPs were needed to identify other polymorphisms (Figure 5). Specifically, 6 tag SNPs have been identified by the International HapMap project (www.hapmap.org), where 3 were intronic and the others were exonic. The 3 exonic tag SNPs identified were the *Q356R* (rs1799950), *D693N* (rs4986850), and *E1038G* (rs16941). These three SNPs all have non-synonymous changes and were used in the haplotyping analysis of BRCA1. In epidemiological studies, some of these variants have been identified to be associated with risk. Surprisingly, these are the same variants that have been designated as tag SNPs and represent several *BRCA1* haplotypes by Haploview © (Haploview 3.32, Broad Institute of MIT and Harvard, Boston, MA). Specifically, the *BRCA1 Q356R* (rs1799950) variant appears to be protective (78-80), although null results also have been reported (81, 82). The *BRCA1 D693N* (rs4986850) has been previously studied and allelic difference were found in breast/ovarian cancer cases and controls where results were not statistically significant, but they proposed that this functional variant may predict breast cancer susceptibility (79). The BRCA1 *E1038G* polymorphism has not been examined independently, but it should be noted that HapMap reveals it is highly linked to other SNPs such as *P871L*, *K1183R*, and *S1613G*, where they are represented in the same haplotype block. However, in the Nurses' Health Study, the 871L variant was not found to be associated with an increased risk of breast cancer (81). Several other associations have been found in subjects with specific BRCA1 mutations and hereditary breast cancer cases with the *K1183R* and *S1613G* genotypes had

differential risks compared to controls (p=0.03 and 0.08 respectively) (83). In this study, the *BRCA1* polymorphisms *Q356R*, *D693N*, and *E1038G* were chosen for investigation because evidence shows these SNPs could be associated with risk. *BRCA1*haplotypes have received some attention; the above three SNPs with comprised of 5 haplotypes as reported by the International HapMap Project. Thus, these SNPs also were used for haplotype analysis.

Figure 5. BRCA1 haplotype LD plot in Caucasians determined by HapMap and computed by Haploview.



Rad51

Rad51 has been proposed as a risk modifier in *BRCA1* mutation carriers because of its interactions with BRCA1 during homologous recombinational (HRR) DNA repair (84-88). *Rad51,* a *rec A* homologue of *E. coli*, has been mapped to chromosome 15q15.1 in humans and

spans more than 39kb. It contains 10 exons and encodes a 339 amino acid protein (genomic accession no: NM_133487).

Rad51 is the central HRR protein in eukaryotes and it catalyses strand transfer between a broken sequence and its undamaged homologue to allow re-synthesis of the damaged region (49). Rad51 is a small protein (37kD) and is biochemically characterized as an ATPase, responsible for homologous pairing and strand exchange, forming nucleoprotein filaments. Specifically, Rad51 binds BRCA2 through 8 BRC repeats that appear to be redundant (89, 90). Rad51, along with BRCA1 and BRCA2, co-localize to irradiation induced foci (IRIF) and, notably, cells deficient in BRCA1 and BRCA2 are also defective in Rad51 IRIF formation (91-93). Additionally, because *BRCA2* carries with it the nuclear localization signal, Rad51 cannot localize to the nucleus without BRCA2 and this has been shown in BRCA2-deficient cells (92). BRCA2 eventually controls the transport of Rad51 and, ultimately, promotes and regulates the homologous recombination pathway of DNA double-strand-break repair. After, nuclear localization, Rad51 protein coats DNA substrates to form nucleoprotein filaments and forms foci in the nucleus after irradiation. Finally, it catalyzes strand exchange forming intermediates in homologous strand pairing (94) that result in the formation of heteroduplex DNA molecules (95). The invasion displaces the non-complimentary intact strand. In the end, enzymatic resolution of the cross-stranded Holliday junction occurs. It has also been shown that loss of Rad51 predisposes cells to genetic instability, leading to chromosomal aberrations and ultimately cancer (96-99).

Little information is known about *Rad51* SNPs and cancer risk, but several epidemiological studies of *Rad51* have been done in *BRCA1* and *BRCA2* mutation carriers.

While some have found positive associations in *BRCA1* mutation carriers (84, 85, 88, 100), no associations have been found in sporadic breast cancer case-control studies (101, 102). A recent study has shown that the *Rad51 5'UTR* variant 135C allele was associated with a decreased risk of breast cancer in *BRCA1* 5382insC mutation carriers (100, 103). While examining *Rad51* genotypes and haplotypes, no SNPs were found in the coding region so haplotyping of was not done. Additionally, the Haploview software chose tag SNPs identified by HapMap were non-coding and found in untranslated or intronic regions and this project's interests were possible functional polymorphisms found in the coding region. It also appears that SNPs in this gene appear to be in high linkage disequilibrium (Figure 6).

Although the functional consequences of the 135G>C polymorphisms still remain unknown, it is speculated that because of its location, it may function in gene expression regulation and may alter mRNA levels (104, 105). Nevertheless, researchers have failed to reach a consensus regarding the functional effects of this *Rad51* polymorphism. Because of BRCA1 and Rad51's combined function, it was proposed that polymorphisms in both genes could consequently lead to decreased DNA repair capacity, especially in individuals whose DNA repair may already be compromised. Although few have reported SNPs in the coding region of *Rad51*, it is believed that in our population of *BRCA1+* subjects, new polymorphisms will be discovered that would predispose them to deficient DNA repair or polymorphisms that compensate for *BRCA1* mutation-associated DNA repair deficiency. A list of *Rad51* polymorphisms, exon functions, Rs numbers, base and amino changes found were compiled from the NCBI (http://www.ncbi.nlm.nih.gov/) website and can be seen in Table 3.

Figure 6. *Rad51* LD Plot for Caucasian population determined by HapMap and computed by Haploview.

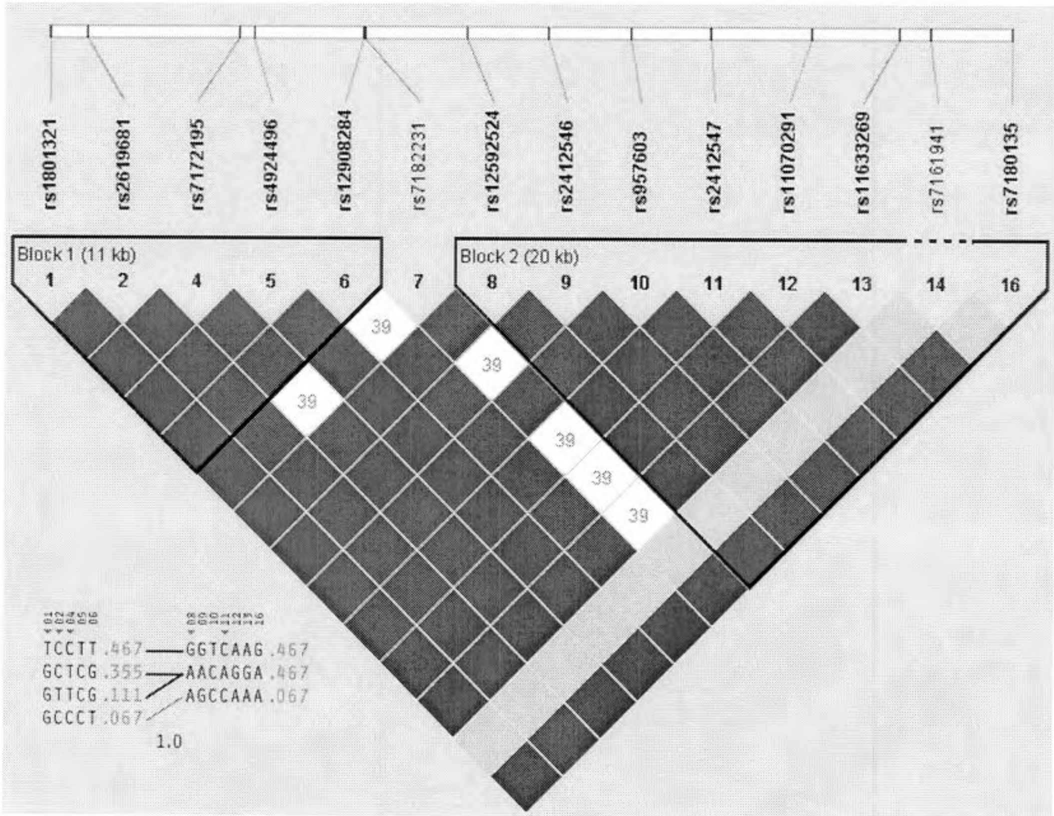


Table 3. Rad51 functions and variants found in each exon.

Exon	Functions	Variants (rs#)	SNP type	Base change	AA change
Promoter	has Sp1 sites	2619679			
Promoter	Has large CpG island	7171780			
Promoter		7171963			
Promoter		5030790			
1		1801320	untranslated	C>G	
1		1801321	untranslated	G>T	
1		2619681	untranslated	C>T	
1		2619680	untranslated	C>A	
1		3092981	untranslated	C>T	
2		NONE			
3	contains HhH DNA binding domain	NONE			
4	contains DNA binding domain/ binds Rads	7174493	non-synonimous	G>C	Val>Leu
5	modulates ATPase activity	NONE			
6	Binds other RAds	NONE			
7	Binds other RAds	11544204	synonimous	G>A	Arg>Arg
7	Bind BRC repeats of BRCA2	7161941	intronic	T>A	
7	Binds DNA	7183120	intronic		
8 and 9	Bind BRC repeats of BRCA2	2229876	intronic	T>A	
8 and 9	Phosphorylated by ABL	4423392	intronic		
8 and 9		1804269		C>T	
8 and 9		11544205	non-synonimous	T>G	Ser>Ala
10		1056742	non-synonimous	C>A	Q>K
10		12593359	untranslated		
10		7180135	untranslated	G>A	
10		11855560	untranslated		
10		11852786	intronic		

Measuring DNA Repair Capacity

Mutagen Sensitivity Assay and DNA repair

Family history is a well-established risk factor for sporadic breast cancer (106, 107) and suboptimal or deficient repair of DNA damage may explain this finding, in part (20-22, 108-116). The mutagen sensitivity assay (MSA), also known as the radiation-induced G2 chromatid break assay, has been proposed as a phenotypic assay of heritable breast cancer risk for both familial and sporadic breast cancer; we are not aware of published null studies using the MSA (mutagen sensitivity assay) for predicting breast cancer risk (20-22, 24, 25, 117-123). This assay

measures the number of chromosomal breaks in cultured lymphocytes following *in vivo* exposure to DNA damaging agents. Different mutagens such as bleomycin, x-rays, and UV light have been used for various cancers, and consistently the MSA (mutagen sensitivity assay) has been shown to be a predictor of many types of cancer, even in small studies (27, 124-137). Gamma radiation has been the most widely used mutagen in breast cancer studies because it is a direct DNA damaging agent that is not dependent of cell penetration, metabolism, or clearance and it is known to trigger a variety of DNA damage. Table 4 lists several studies of the MSA (mutagen sensitivity assay) in breast cancer patients, family members, and controls.

Table 1. Mutagen Sensitivity in healthy controls compared to breast cancer cases and family members.

Cases MBPC	Control MBPC	Family MBPC	Source of radiation/ Mutagen	Dosage	Gaps and/or Breaks	Post-irradiation Incubation (Hours)	References
0.61±0.24	0.45±0.14		γ-rays	1.25 Gy	Breaks	5	(24)
1.1-1.64	0.16-0.5		X-rays	0.58Gy	Gaps and breaks	1.5	(20,117)
0.82±0.18 (Sporadic) 1.28±0.44 (Familial)	0.58±0.20	0.79±0.22	Bleomycin	0.03 units/ml	Gaps and breaks	-	(108)
1.1-1.9	0.30-0.40	0.38-0.49	X-rays	100/58R	Gaps and breaks	0.5	(109)
0.91-2.91	0.57-1.5	1.36	X-rays	0.58Gy	Gaps and breaks	0.5	(22)
1.38	0.38	0.42 (spouse)	X-rays		Gaps and breaks	0.5	(23,116)
0.85±0.37	0.63±0.19		γ-rays	0.4	Breaks	0.5	(118)
0.8-2.6	1.09		X-rays	0.58Gy	Gaps and breaks	1.5	(122)

Because the MSA (mutagen sensitivity assay) has been shown to be a heritable trait, it has been used to study breast cancer in high-risk families (20-22, 108-116). For example, in a twin study, Wu and colleagues identified a high concordance among monozygotic twins and a lower concordance among dizygotic twins and again showed that mutagen sensitivity is a heritable trait using several DNA repair assays (115). In a comparison of mutagen sensitivity in hereditary breast cancer cases, their family members and sporadic breast cancer cases, it was found that 97% of hereditary breast cancer cases, 30% of their family members and, 44% of sporadic cases were sensitive (>0.8 mean breaks per cell) to Bleomycin, another mutagen frequently used in the MSA (mutagen sensitivity assay) (108). In another study of cancer-prone

individuals, individuals with a family history of breast cancer, it was found that their irradiated cells displayed a two-fold higher number of breaks compared to healthy controls (116). Additionally, it was found that women at high risk (having 1 or more first-degree relative with breast cancer) were 5 times more likely than controls to have suboptimal DNA repair (OR=5.2, 1.04-28.57) and that deficient DNA repair was found in all women with breast cancer, but in only 32% of control women (p=0.02) (20). Therefore, there is much evidence that support the hypothesis that the MSA (mutagen sensitivity assay) is a good biomarker for decreased DNA repair.

Several studies have used DNA repair assays in *BRCA1* and *BRCA2* mutation carriers to assess chromosomal instability and to demonstrate relationship to breast cancer risk. However, results have been inconsistent, probably due to small numbers of patients and the type of assay performed (MSA and Micronuclei Assay) (138-140). By assessing radiation-induced MN formation, *BRCA1* mutation carriers showed elevated radio-sensitivity compared to unaffected controls; however, no difference was seen between *BRCA1* mutation carriers and unaffected controls post irradiation using the comet assay (140). These studies were significant because DNA repair functional assays were used to determine repair capacities in subjects with variant DNA repair genotypes. Again, this can be considered development of *a priori* hypotheses because theoretical and plausible evidence was used to choose genes to be looked at in genotype-phenotype correlations.

Defective break repair has also been shown to be a consequence of several other polymorphic genes in several genotype-phenotype association studies (Table 2). For example, Lunn et al showed that the L715L SNP was associated with suboptimal DNA repair compared to

L715Q with an OR of 7.2 (95%CI: 1.01-87.7) (141). In *XRCC,* 2 SNPs (R280H and R399Q) have been found to be associated with DNA repair capacity. Additionally, polymorphisms in the *XPD* and XPC, showed correlation to deficient DNA repair (141, 142). In a more recent study, Lu et al hypothesized that genes involved in DNA repair, *Rad51* and P53, could alter risk of squamous cell carcinoma. Results indicated that a combination of SNPs were associated with decreased gamma radiation-induced chromatid breaks (143) Other studies evaluating genotypes and DNA repair capacity can be viewed in Table 2 and can be found in a review by Hu et al (144). Because, again, the relationship between a SNP and function is usually unknown, *a priori* hypotheses must be established to increase the likelihood of finding true-positive associations (17, 18). In the studies described above, the candidate-gene and candidate-pathway approach was used in this study to develop an *a priori* hypothesis.

Table 2. Genotype-Phenotype studies of DNA repair Capacity.

Gene	Results	Reference
XPD	MSA found Lys/Lys associated w/ suboptimal repair OR=7.2 95%CI=1.01-87.7	(141)
XPC	Irradiation specific DNA repair rates were affected by the K939Q polymorphism	(142)
XRCC3	MSA and SCE found 241met associated with decreased breaks in healthy caucasians OR=0.5 (0.16,0.84)	(194)
XRCC1	MSA found 280 variant allele displayed 28% higher mean breaks per cell	(195)
XRCC1	Mean SCE among current smokers with 399Gln allele greater than 399Arg/Arg	(193)
Rad51 and P53	Rad51 SNP associated with fewer gamma radiation-induced MBPC	(143)

Mutagen Sensitivity Assay Technical Considerations

Most studies apply the MSA (mutagen sensitivity assay) to freshly cultured blood, although some studies have used cryopreserved lymphocytes (136, 145). The requirement for fresh blood cultures is constraining on epidemiological studies because the availability of subject samples are unpredictable. Thus, the use of archived immortalized cell lines has the following

advantage of providing an endless amount of sample. Immortalization of cell lines by EBV is useful for preserving valuable specimens and it allows for the retrospective assessment of archived blood samples, which exist for many epidemiological studies. Although, cryopreserved lymphocytes have been used when fresh sample is not available, cryopreserved cells do not retain the same repair capacity for culture as fresh cells, and so this might also affect MSA (mutagen sensitivity assay) results. A mutagen sensitivity comparison using an assessment of micronuclei formation following radiation exposure of lymphocytes in fresh blood and cryopreserved lymphocytes showed a poor correlation (146). Additionally, other studies show that cryopreserved cells are potentially more sensitive to mutagen exposure in groups of study subjects (136, 145).

These shortcomings may be circumvented by immortalizing or transforming and continuously growing the cells under investigation. Human B lymphocytes can be immortalized with infection by EBV (Epstein-Barr Virus) *in vitro* as well as *in vivo*. Immortalizing the cells with EBV provides cell lines for future studies. EBV is able to immortalize resting B cells and allow lymphoblastoid cell lines (LCLs) to proliferate continuously, ultimately leading to an endless supply of monoclonal B-cell lymphocytes. With EBV immortalization, a culture is capable of greater than 150-200 doublings and is considered immortal. Although genetic alterations may occur, these genetic changes presumably provide the affected cell with either proliferative or survival advantages, and may play an important role in the *in-vitro* establishment of the cell line (123, 147). It should be noted that PHA-stimulated lymphocytes in freshly cultured blood stimulates T-cell lymphocyte proliferation, and so the MSA (mutagen sensitivity assay) applies to these cells, while EBV-immortalized cells are mostly B-cell lymphocytes. Thus,

the different cell types might affect the predictive capability for the MSA (mutagen sensitivity assay) assay. To date, no studies directly comparing the MSA (mutagen sensitivity assay) for freshly cultured cells and concordant EBV immortalized cells in the same study subjects, so that it remains unknown if prior results are due to differences in study subject or assay results.

SUMMARY

The relationship between DNA repair genotypes and phenotypes are still actively being investigated and attempts to draw a correlation between inheritance of a particular mutation or SNP and the resulting DNA repair phenotype are only now being explored. To understand the relationship between DNA repair and genetic susceptibility, the present study will investigate the association between specific *BRCA1* SNPs (*Q356R*, rs1799950; *D693N*, rs4986850; *E1038G*, rs16941) and haplotypes, *Rad51* SNPs, and deficient DNA repair.

Body

Task 1: To compare the number of induced chromosomal breaks in EBV-immortalized and freshly cultured lymphocytes from affected and unaffected *BRCA1* carriers.

Aim 1: To establish and validate the mutagen sensitivity assay (MSA) and compare the number of induced chromosomal breaks in EBV-immortalized and freshly cultured lymphocytes from high risk breast cancer families.

Hypothesis: Mutagen sensitivity in freshly cultured lymphocytes will be correlated with mutagen sensitivity in immortalized lymphocytes from the same women.

Validation of the Mutagen Sensitivity Assay

Several preliminary experiments were performed to test the conditions of the mutagen sensitivity assay on EBV-immortalized cell lines. Figure 1 shows a diagrammatic representation of a few types of gamma radiation-induced chromosomal damage. To test if conditions of the mutagen sensitivity assay had to be changed from the original PBL conditions, a hypotonic solution time response test (Figure 1), colcemid dose and time response test (Figure 2), and radiation dose response test were performed (Figure 3 and 4). It was demonstrated that 1) 35 min hypotonic solution treatment was ideal; 2) 10ug/ul Colcemid solution at 1.5 hours provided the best metaphases and revealed best information; and 3) 1 Gray treatments were best because at 2 and 4 Gray, no metaphases were apparent.

Figure 1. a) Diagrammatic representation of a few types of gamma radiation-induced chromosomal damage b) The 35 and c) 45 minute treatment of 0.06 potassium chloride.

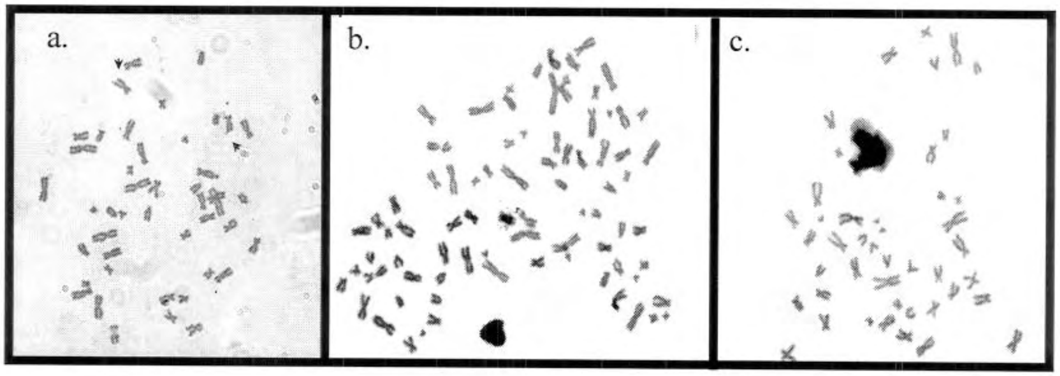


Figure 2. a) A dose of 10ug/uL at 2h and b) 20ug/uL. c) A dose of 10ug/uL at 1.5 hours.

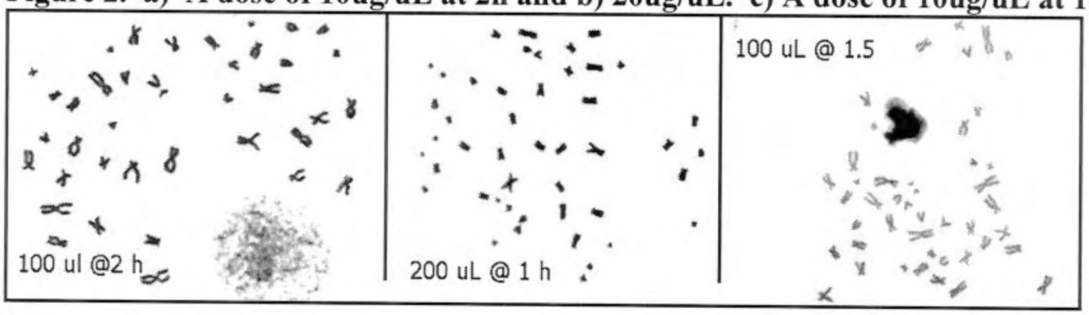


Figure 3. Metaphases at a) 0Gy b) 1 Gy c) 2 Gy and d) 4 Gy.

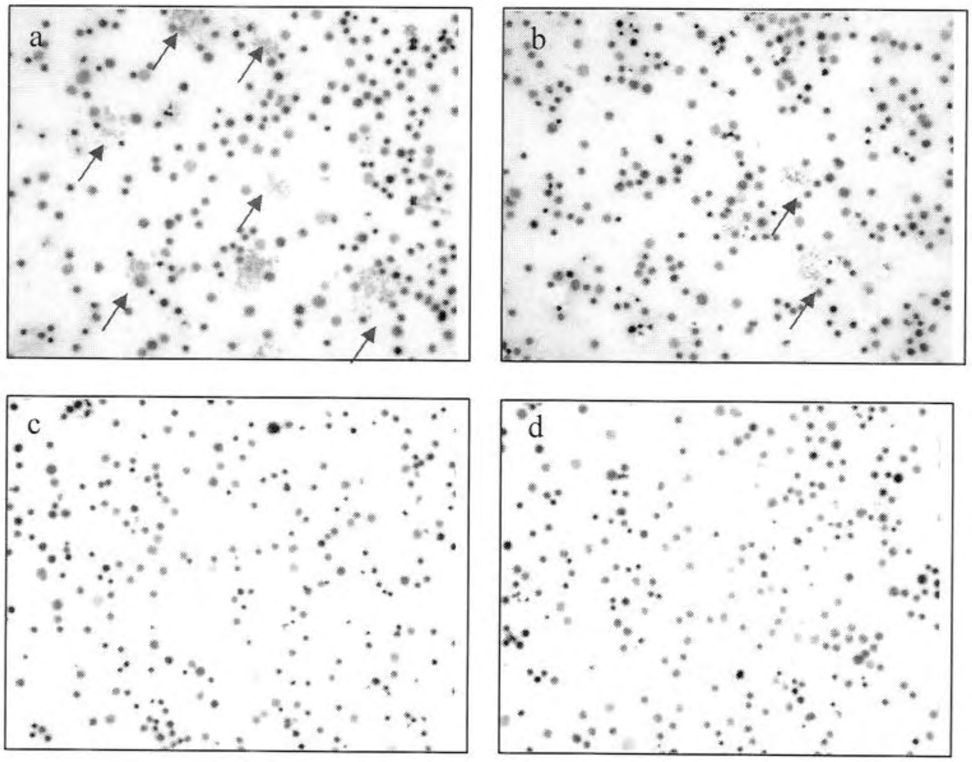
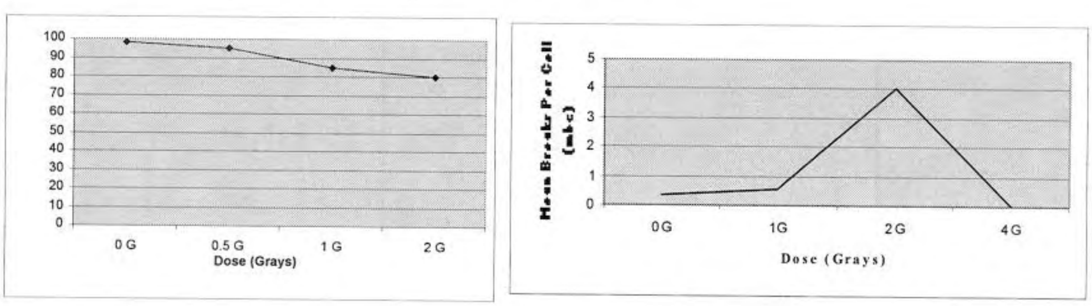


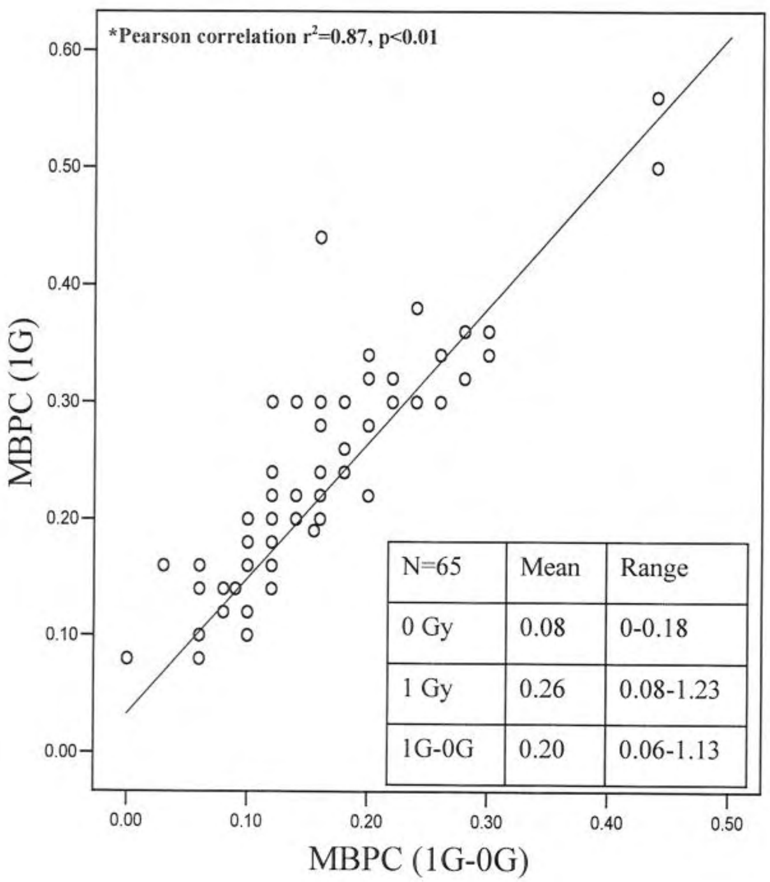
Figure 4. a) Percentage cell survival at different quantities of radiation. b) Mean breaks per cell (MBPC) at different quantities of radiation.



Initial experiments were performed to determine if the difference for MBPC (mean breaks per cell) of irradiated to un-irradiated baseline cultures provided different results than the MBPC (mean breaks per cell) of irradiated cells. This was tested in 65 subjects using the EBV-immortalized cell lines. It was found that there was strong correlation between the difference of

MBPC (mean breaks per cell) for irradiated minus control cultures and the absolute MBPC (mean breaks per cell) for irradiated cultures ($r^2=0.87$, $p<0.01$), probably because the absolute MBPC (mean breaks per cell) for baseline cultures is low compared to the irradiated cells (Figure 5). Thus, for future experiments, baseline MBPC (mean breaks per cell) were not counted.

Figure 5. Irradiated cell MBPC (mean breaks per cell) highly correlated with difference.



Comparison of slide readings between cytogeneticists

During preliminary experiments, cytogenetic training was provided by an expert cytogeneticist in the lab (Dr. Ramona Dumitrescu), who had previously trained in the laboratory of Dr. Theodore Puck and has participated in other inter-laboratory comparisons of the assay. Following the training, a set of slides were blindly read by Dr. Dumitrescu and myself, to test inter-observer variability. Fifteen slides were scored blinded to each others scores, subject status, and treatment conditions. Results from that experiment are described in the Table 1, showing a strong correlation in readings of same samples between cytogeneticists (ρ=0.97, 95%CI= 0.91-0.99; $p<0.01$). The mean and standard deviation for reviewer 1 (LRS) and 2 (RD) was 0.21 +/-0.12 and 0.22 +/- 0.14, respectively. The CV (57.04%) and intra-individual variation was calculated and are listed on Table 2. The *t*-tests was used here to compare the means and it showed that the means between the two reviewers were not significantly different (p=0.93; Table 1).

Table 1. Comparison of slide readings between reviewer 1 and 2.

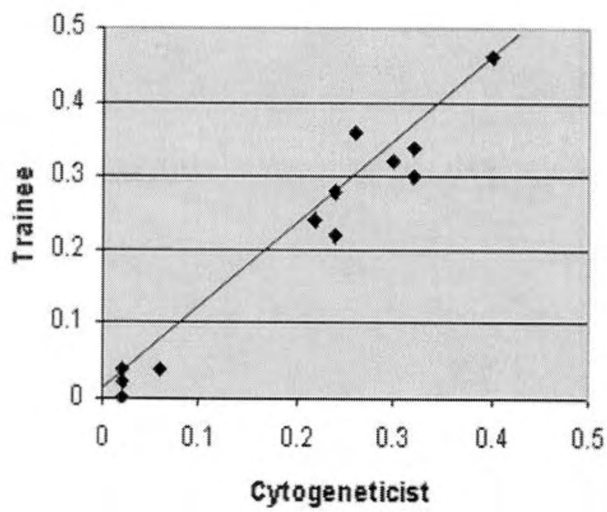
Subject ID	Reviewer 1 (MBC)	Reviewer 2 (MBC)	Mean (SD)	CV (%)
1	0.24	0.28	0.26 (0.02)	7.69
2	0.24	0.22	0.23 (0.01)	4.35
3	0.24	0.22	0.23 (0.01)	4.35
4	0.32	0.30	0.31 (0.01)	3.23
5	0.24	0.22	0.23 (0.01)	4.35
6	0.24	0.22	0.23 (0.01)	4.35
7	0.26	0.36	0.31 (0.05)	16.13
8	0.40	0.46	0.43 (0.03)	6.98
9	0.22	0.24	0.23 (0.01)	4.35
10	0.30	0.32	0.31 (0.01)	3.23
11	0.32	0.34	0.33 (0.01)	3.03
12	0.02	0.04	0.03 (0.01)	33.33
13	0.02	0.02	0.02 (0.00)	0
14	0.02	0.00	0.01 (0.05)	100
15	0.06	0.04	0.05 (0.01)	4.43
Mean (SD)	0.21 (0.12)	0.22 (0.14)		

*Spearman correlation $\rho = 0.97$; 95%CI=0.91-0.99; $p < 0.01$

**T-test $p=0.93$

***CV (%) = 57.04

Figure 6. Comparison of slide readings between Cytogeneticist and Trainee.

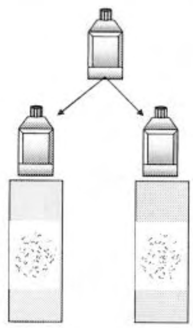


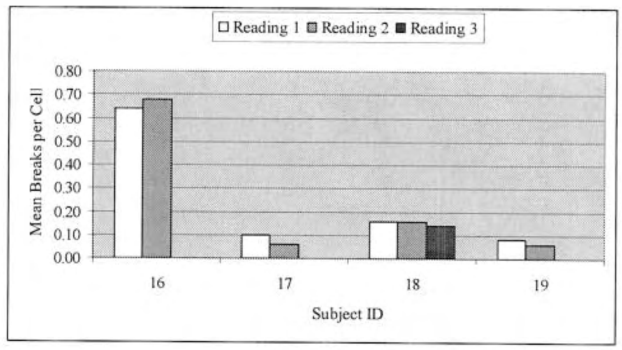
Intra- and Inter-individual variation assessment

Assay variability was assessed for EBV-immortalized cell cultures by determining the MBPC (mean breaks per cell) in replicates cultured on the same day (different aliquots of the same individuals; Figure 7a). It was found that replicate readings from the same aliquot (n=4 subjects) had a rho value of 0.91 (95%CI=-0.41-0.99; $p<0.01$; Table 2a and Figure 7a). The CV (56.37%) and intra-individual variations were calculated and are listed on Table 2a. Again, the *t*-tests was used to compare the means and it showed that the means between the readings were not significantly different ($p=0.26$). Then, replicate EBV-immortalized cultures from 10 more subjects were thawed and cultured for different lengths of time, 3 days apart. Specifically, 10 paired cultures were set up and 3 days later, one aliquot was used for the metaphase preparation and the other aliquot remained in culture for 3 more days after replacing the old media. Results were then compared using the spearman correlation ($\rho=0.96$, 95%CI=0.84-0.99; $p<0.01$) (Table 2b and Fig.7b). The CV (85.52%) and intra-individual variations were calculated and are listed on Table 2b. A *t*-test also confirmed that there were no significant differences between readings ($p=0.84$). ANOVA was also done to determine variance within each sample and to determine if there were differences in the methods and it was found that there were no difference within each sample ($p=0.8237$). In the end, these validation studies confirm that 1) cytogenetic student can give reliable results and that 2) mutagen sensitivity assay was reproducible in EBV immortalized cell lines.

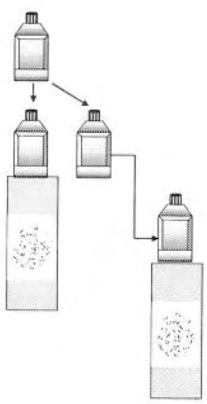
Figure 7. Mean breaks per cell of EBV-immortalized cell lines when a) replicates cultured on the same day and b) on different days (different aliquots).

a.





b.



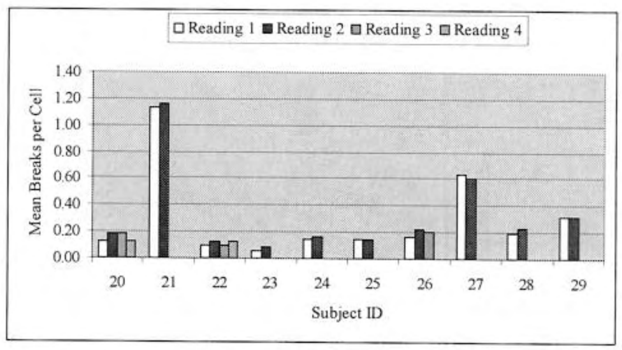


Table 2. a) Comparison of replicates cultured on the same day and b) for different lengths of time (different aliquots).

a)

Subject ID	Readings (MBC) 1	2	3	Mean (SD)	CV (%)
16	0.64	0.68		0.66 (0.02)	3.03
17	0.10	0.06		0.08 (0.02)	25.00
18	0.16	0.16	0.14	0.15 (0.01)	4.35
19	0.08	0.06		0.07 (0.01)	14.29

*Spearman correlation ρ= 0.91; 95%CI=-0.41-0.99 and correlation is significant at 0.01 level.
***T*-test= 0.036, *p*= p=0.26
***CV (%) = 56.41

b)

Subject ID	Readings (MBC) 1	2	3	4	Mean (SD)	CV (%)
20	0.12	0.18	0.18	0.12	0.16 (0.03)	23.09
21	1.13	1.16			1.15 (0.02)	1.85
22	0.10	0.12	0.10	0.12	0.11 (0.01)	10.50
23	0.06	0.09			0.08 (0.02)	28.28
24	0.14	0.16			0.15 (0.01)	9.43
25	0.14	0.14			0.14 (0.00)	N/a
26	0.16	0.22	0.20		0.19 (0.03)	15.80
27	0.63	0.60			0.62 (0.02)	3.45
28	0.19	0.23			0.21 (0.03)	13.47
29	0.32	0.32			0.32 (0.00)	N/a

*Spearman correlation ρ= 0.96; 95%CI=-0.84-0.99 and correlation is significant at 0.01 level.
***T*-test= -0.206, *p*=0.84
***CV (%) = 106.66

Comparison of fresh whole blood cultures and EBV-immortalized lymphoblasts

Nineteen women provided fresh blood samples for MSA (mutagen sensitivity assay), and an aliquot was used to prepare EBV-immortalized lymphoblasts. The MSA (mutagen sensitivity assay) was performed on the fresh blood, and then later on the EBV-immortalized lymhoblasts after they were established and frozen. The MBPC (mean breaks per cell) in EBV-immortalized cell lines was significantly correlated with the MBPC (mean breaks per cell) in freshly cultured lymphocytes from the same subjects (ρ=0.92, 95%CI=0.79-0.97; p<0.01). The results are

summarized in Table 3 and are presented graphically in Figure 8. The MBPC (mean breaks per cell) were 0.27 +/- 0.14 and 0.29 +/- 0.13 for PBL and EBV-immortalized lymphocytes, respectively and the *t*-test was used to measure difference in the means (p=0.11; Table 4). The CV (46.03%) and intra-individual variations were calculated and is listed on Table 4.

Table 3. Descriptive statistics for peripheral blood lymphocytes and EBV-immortalized cell lines.

	N	Minimum	Maximum	Mean	Std. Deviation
PBL	19	0.1	0.62	0.27	0.14
EBV	19	0.1	0.56	0.29	0.13

Spearman correlation ρ= 0.92 and correlation is significant at 0.01 level.

Figure 8. Scatter plot of paired peripheral blood lymphocytes and EBV-immortalized cell lines. Solid line= best fit line

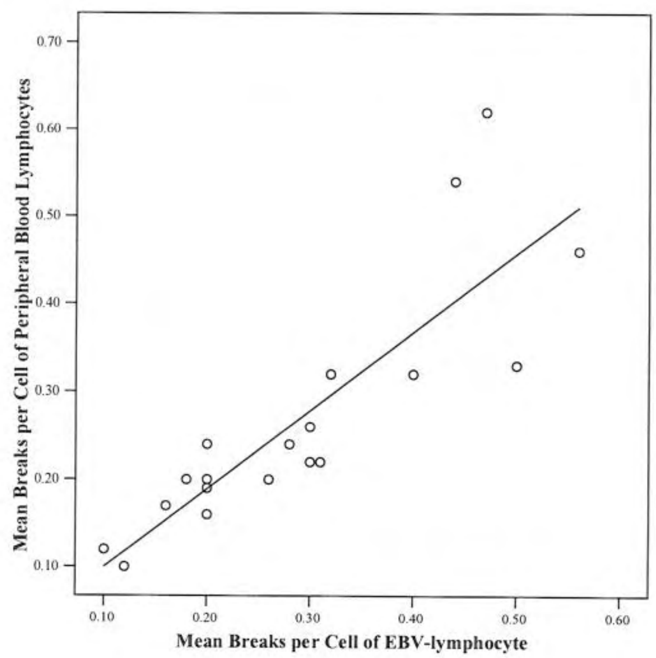


Table 4. Comparison of Mean breaks per cell in peripheral blood lymphocytes and concordant EBV-immortalized cell line irradiated with 1 Gray. MBPC (mean breaks per cell)

Subject ID	Blood (MBC)	EBV (MBC)	Mean	CV (%)
30	0.12	0.10	0.11 (0.01)	9.09
31	0.26	0.30	0.28 (0.02)	7.14
32	0.16	0.20	0.18 (0.02)	11.11
33	0.32	0.40	0.36 (0.04)	11.11
34	0.10	0.12	0.11 (0.01)	9.09
35	0.46	0.56	0.51 (0.05)	9.80
36	0.24	0.20	0.22 (0.02)	9.09
37	0.22	0.30	0.26 (0.04)	15.38
38	0.20	0.26	0.23 (0.03)	13.04
39	0.19	0.20	0.20 (0.01)	2.56
40	0.17	0.16	0.17 (0.01)	3.03
41	0.32	0.32	0.32 (0.00)	0.00
42	0.33	0.50	0.42 (0.09)	20.48
43	0.62	0.47	0.55 (0.08)	13.76
45	0.24	0.28	0.26 (0.02)	7.18
46	0.20	0.18	0.19 (0.01)	3.59
47	0.22	0.31	0.27 (0.05)	16.15
48	0.54	0.44	0.49 (0.05)	17.95
49	0.20	0.20	0.20 (0.00)	0.00
Mean (SD)	**0.27 (0.14)**	**0.29 (0.13)**		

*Spearman correlation ρ= 0.92; 95%CI=-0.79-0.97; p< 0.01
***T*-test p=0.11
***CV (%) = 46.03

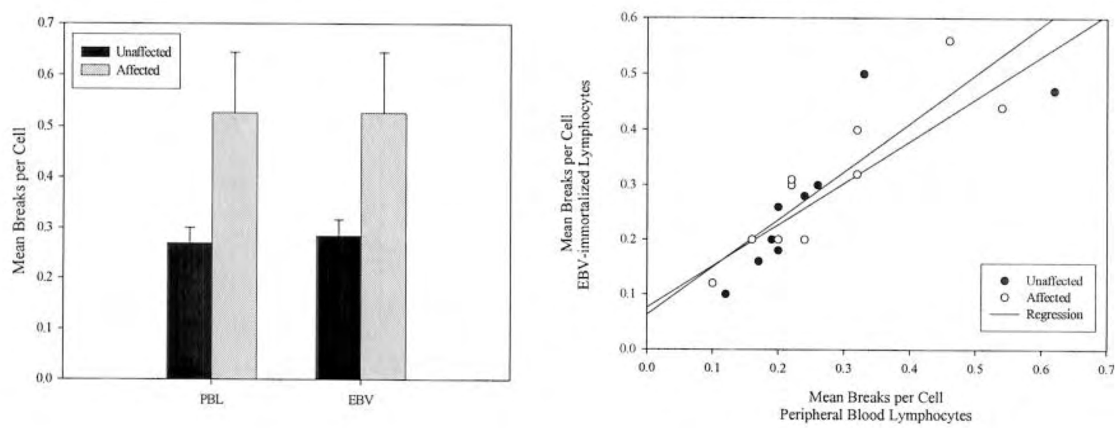
The results were also analyzed separately by cancer status (affected with a history of breast cancer or unaffected and no history of breast cancer) to determine if the presence of cancer could affect the validation (Table 5 and Figure 9). In this small sample set, it was found, that the unaffecteds did not have differential MBPC (mean breaks per cell) compared to affecteds for freshly cultured blood (0.26 +/- 0.15 and 0.28 +/- 0.14, respectively; p=0.77) and EBV-immortalized lymphocytes (0.31 +/- 0.14 and 0.27 +/- 0.13, respectively; p=0.53). Additionally, cancer status did not affect the correlation for the comparison of freshly cultured blood and EBV-immortalized lymphocytes. The rho value was 0.91 (95%CI=0.62-0.98; p<0.01) in the affecteds and rho was 0.95 (95%CI=0.79-0.99; p<0.01) for the unaffecteds.

Table 5. Descriptive statistics of MBPC (mean breaks per cell) for affected (with cancer) and unaffected (without cancer) peripheral blood and EBV-immortalized cell lines

	N	Minimum	Maximum	Mean	Std. Deviation
Affected PBL*	10	0.1	0.54	0.28	0.14
Affected EBV*	10	0.12	0.56	0.31	0.13
Unaffected PBL**	9	0.12	0.62	0.26	0.15
Unaffected EBV**	9	0.1	0.5	0.27	0.14

*Spearman correlation ρ=0.91 (95%CI=0.62-0.98) and correlation is significant at 0.01 level.
**Spearman correlation ρ=0.95 (95%CI=0.79-0.98) and correlation is significant at 0.01 level.

Figure 9. Peripheral blood lymphocytes and EBV-immortalized cell line MBPC (mean breaks per cell) (mean breaks per cell) stratified by cancer status. a) Bar graph shows that affected subjects will always have more mean breaks per cell. b) Spearman correlation for unaffected and affected is 0.95 and 0.91, respectively.



Task 2: To study DNA repair proficiency in the terms of chromosomal break and gap frequencies in EBV-immortalized lymphocytes of *BRCA1*-related breast cancer patients and unaffected carriers following *in vitro* treatment with γ-irradiation.

Aim 2: To identify DNA repair phenotypes in affected (women with cancer) and unaffected (women without a history of cancer) high-risk individuals from breast cancer families by using the mutagen sensitivity assay that determines the resultant chromosomal breaks in EBV-immortalized lymphocytes following *in vitro* treatment with gamma irradiation.

Hypothesis 2a: There will be a range of responses, i.e. inter-individual variation, for chromosomal breaks using gamma irradiation among high-risk individuals from breast cancer families.

Hypothesis 2b: There will be greater MBPC (mean breaks per cell) in high-risk individuals from breast cancer families that have a history of breast cancer compared to those who do not.

Mutagen Sensitivity Assay Results:

To determine functional relevance of SNPs on DNA repair efficiency, we analyzed gamma radiation-induced chromatid breaks per cell (mutagen sensitivity) in 138 subjects and investigated the modulating effect of SNPs of *BRCA1* and *Rad51*. Subject Demographics are described in Table 6 and Figure 10. The mean age of the study subjects for women without breast cancer (unaffected) was 47.44 (SD=13.63) and with breast cancer (affected) was 44.73 (SD=10.65). The age ranges of the unaffected and affected subjects were 25-78 and 27-79, respectively. Of those 138 cell lines obtained, 117 were BRCA1 mutation carriers (73 affected and 44 unaffected). There were 40 women of Ashkenazi Jewish descent with the 185delAG founder mutation and 16 with the 5382insC founder mutation. Among the women without a

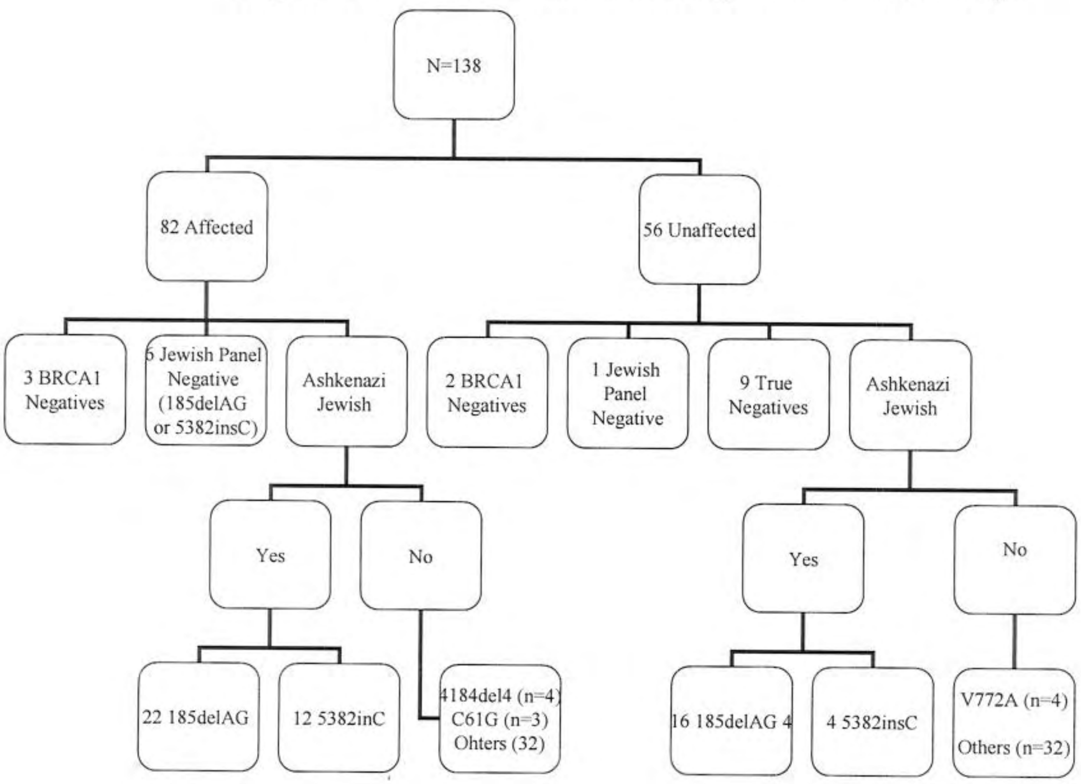
detected mutation, 5 were BRCA1/2 negative, 7 women were negative for the 185delAG and 5382insC mutation, and 9 were true negatives (women with a positive family member). In this population, 48 (34.78%) subjects were related.

Table 6. Subject demographics.

	Unaffected	Affected	*P**
	N=56	*N=82*	
Age Range (yrs)	25-78	27-79	
Mean Age (yrs)	47.44	44.73	0.43
Median Age (yrs)	50	45	
Standard Deviation	13.63	10.65	
Mutation			
185delAG (BRCA1)	19 (31.6%)	21 (24.7%)	
5382insC	4 (7.0%)	12 (14.8%)	
Other	35 (61.4%)	49 (60.5%)	

**P* values for two-sided

Figure 10. Characterization of Subjects in current study.



Of the 138 subjects, 43 (31%) did not have 50 metaphases. Specifically 41 (73%) of the unaffecteds and 54 (66%) of the affecteds had 50 metaphases. However, subjects with less than 50 metaphases were not more likely to be affected ($\chi 2=0.22$, p=0.64) or have high MBPC (mean breaks per cell) ($\chi 2=0.71$, p=0.39).The distribution of breaks in all subjects is described in Figure 11. The range of MBPC (mean breaks per cell) overall was 0.02-2.05 MBPC (mean breaks per cell), and for affected cases and unaffected cases it was 0.02-0.75 and 0.04-2.05, respectively.

In unrelated subjects (n=110), affected subjects with breast cancer were 2 times more likely to have higher mean breaks per cell than women without breast cancer, (OR=2.0, 95% CI: 0.9-4.6; $\chi^2 = 3.02$, p=0.10) (Table 7). When subjects were included in the analysis (n=138), the OR increased and results became significant (OR=3.2 95%CI: 1.5-6.7; $\chi^2 = 7.1$, p=0.0077) (Table 8). Figure 12 illustrate the shift in the curves showing affected had higher MBPC (mean breaks per cell).

Table 7. Relationship between Mutagen Sensitivity and Cancer Status in unrelated individuals.

	Mutagen Sensitivity (MBC)			
Mean Breaks per Cell	Unaffected (N=35)	Affected (N=76)	OR	95% CI
Low *	20	30	1.0	
High	15	46	2.0	0.9 - 4.6

$\chi 2= 3.02$, p=0.10 * Low <0.22

Figure 11. Frequency distribution of mean breaks per cell in unaffected (solid line) and affected (dotted line) in unrelated individuals (n=110)

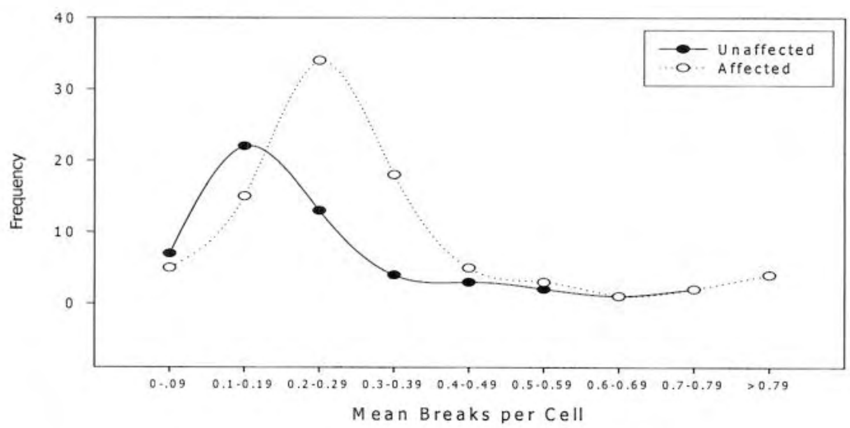
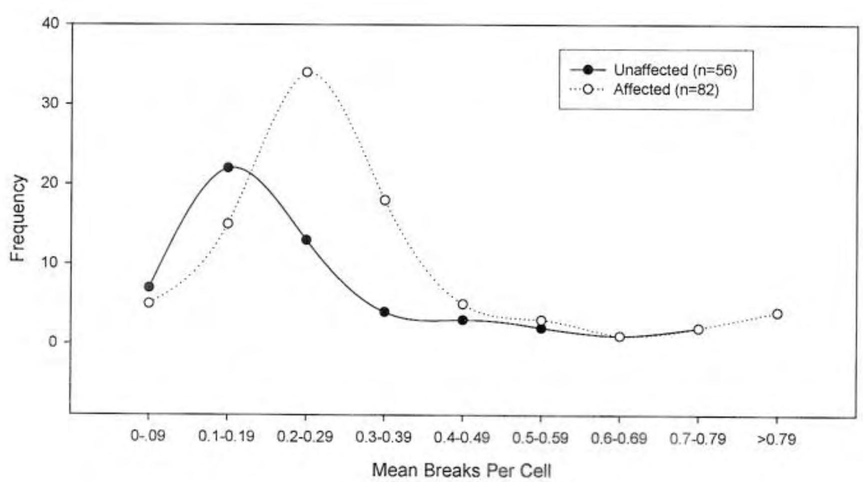


Table 8. Relationship between Mutagen Sensitivity and Cancer Status.

	Mutagen Sensitivity (MBC)			
Mean Breaks per Cell	Unaffected (N=56)	Affected (N=82)	OR	95% CI
Low *	26	18	1.0	
High	30	64	3.2*	1.5 - 6.7

* Low was determined by Unaffected, median MBPC (mean breaks per cell) (<0.18)
** $\chi 2$= 7.1, p=0.0077

Figure 12. Frequency distribution of mean breaks per cell in unaffected (solid line) and affected (dotted line) *BRCA1* mutation carriers (N=138).



Task 3: To identify candidate single nucleotide polymorphisms in genes that encode for *BRCA1*-associted proteins (*NBS1, BRCA2, RAD51, 51B, RAD52, RAD54, 54B, XRCC2*, and *XRCC3*) that affect the phenotype in EBV-immortalized lymphoblasts.

Aim 3: To identify single nucleotide polymorphisms (SNPs) and haplotypes in *BRCA1,* and *Rad51* associated with defective DNA repair capacity in EBV-immortalized lymphocytes.

Hypothesis 3a: There will be polymorphisms and haplotypes in DNA repair genes (*BRCA1* and *Rad51*) that will affect the number of chromosomal breaks in affected and healthy high-risk individuals from breast cancer families.

Hypothesis 3b: There will be gene-gene interactions for specific types of *BRCA1* mutations (Ashkenazi founder mutations – 185delAG and 5382insC) and polymorphisms and haplotypes in DNA repair genes (BRCA1 and *Rad51*) that will affect the number of chromosomal breaks is women stratified by their type of mutation.

Rad51 Sequencing

DNA Isolation Results

When using the Qiagen M48 Biorobot to isolate DNA, we encountered several challenges. This technology has not been previously applied to cell culture studies; the primary use is in the clinical setting for extraction from blood and buccal cells. Using these protocols, the yields included "bad" quality DNA with high concentrations of protein. Later, a cell culture DNA isolation protocol was acquired and it was determined that too many cells were used and the pipets were clogged as a result. Improved yields were obtained when $2.0\text{x}10^6$ or less cells were used.

The phenol-chloroform method for isolation of DNA was successful and DNA concentrations were good (absorbance= 1.7-1.9) to begin sequencing and genotyping analysis.

Sequencing Results

Rad51 exons 2-9 were amplified and sequenced in subjects in the highest and lowest MPBC quartiles (n=79). Sequencing conditions, primers and reagents were used as provided by the Applied Biosystems VariantSEQr Resequencing System for the *Rad51* coding region. With this system, 11 RSAs (**R**e**S**equencing **A**ssays) encompassed 9 exons. Because each primer was flanked by M13 universal primer sequence, the M13 forward and reverse primers were used for all amplifications. Figure 13 demonstrates an example of PCR results on a 2% agarose gel. Subjects with the highest and lowest mean breaks per cell had the *Rad51* coding regions sequenced and Table 9 demonstrates variation found, if the variant was confirmed by reverse sequencing, and if it has been previously published. Figure 14 illustrates several sequences where differences were found.

Figure 13. PCR results of *Rad51* exons 3, 4, 6, and 8.

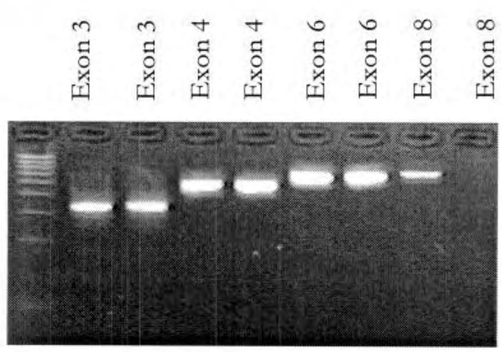
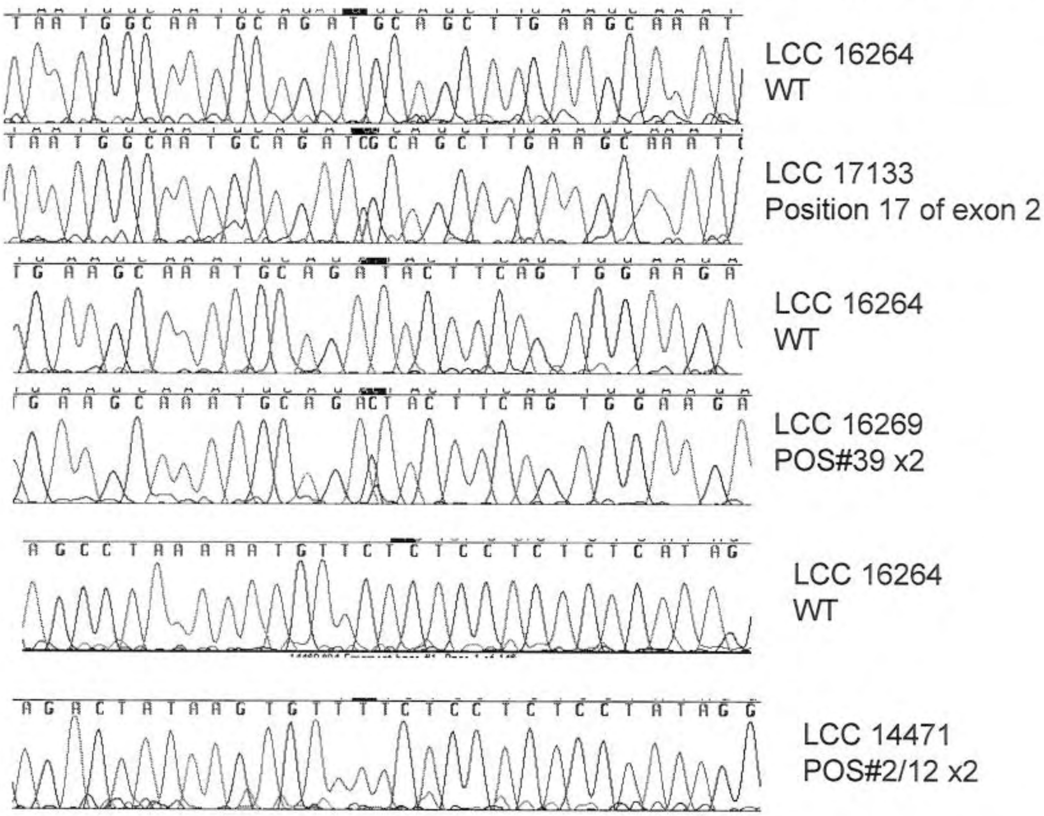


Table 9. Confirmed *Rad51* genetic variation found in coding region

LCC	Polymorphism	Exon
17133	17M	2
16269	39M	2
14471	2M, 12M	2
14726	11Y	3
15984	136M	3
15918	19W	3
15904	77S	3
14464	78Y	3
15896	79W,158W,228W	3
14880	80R	3
15896	11Y,19W	4
16244	138M	4
15926	32W	4
16478	72M	4
16470	**80W**	4
16470	104M	6
16474	91R	6
16269	146W,162R	7
14880	181W	7
15854	187M	7
16237	200R, 250M	7
16484	202M,216N,234S	7
16474	226M	7
16472	260M,276R	7
15913	205R	8
16484	228M	8
14805	24W	8
14804	33W	8
16262	41M	8
16254	46W	8
15896	81M	8
14433	398M/438W,476M	9
16484	460R	9

Figure 14. *Rad51* exon 2 sequences where variation was found.



Although several polymorphisms were discovered and confirmed, none had a frequency of >5% and were not analyzed further. Also, when checked against the NCBI website, none of these were found and they all represent new *Rad51* sequence variants.

Genotype results in high risk subjects

Rad51 135G>C polymorphism analysis

Rad51 135G>C analysis was done by Taqman methods. First, a χ^2 test of observed versus expected genotype frequencies for *Rad51 C>G* among subjects followed Hardy–Weinberg equilibrium ($P = 0.65$) (Table 10).

Table 10. ***Rad51*** **5'UTR 135G>C $\chi2$ analysis and test for Hardy-Weinberg.**

	Observed	Expected	$\chi2$	*p*
GG	0.68	0.68		
CG	0.29	0.29		
CC	0.02	0.031	0.53	0.39

The relationship for *Rad51* 5'UTR 135G>C alleles and genotypes and MBPC (mean breaks per cell) are also reported in Table 11. There was a positive association for *Rad51* 5'UTR 135C allele and higher MBPC (mean breaks per cell) (p=0.03), and the combined homozygous CC genotype and the heterozygote had an age-adjusted OR of 2.5 (95% CI: 1.02-6.13) (Table 11). The unadjusted OR was 3.40 (95% CI: 1.20-9.90).

Table 11. Relationship between Mutagen Sensitivity and *Rad51* 5'UTR 135C>G polymorphisms after removing related individuals from analysis. *Adjusted for age.

Genotype	Low	Hi	OR	95% CI			OR*	95% CI			Wald χ^2	*p*
GG	34	39	1.00									
CC/CG	9	26	3.40	1.20	-	9.90	2.50	1.02	-	6.13	4.29	0.03

BRCA1 genotyping

BRCA1 sequencing was reported by Myriad genetics (Myriad Genetics, Inc., Salt Lake City, UT) for women without Ashkenazi Jewish descent. For the latter, genotyping for founder mutations were done at the Clinical Molecular Diagnostics Laboratory at Georgetown University. The most commonly found SNPs in this population were *Q356R* (22.86%), S2414S (34.29%), *E1038G* (40.0%), *K1183R* (40.0%), L771L (42.8%), S1436S (42.8%), *S1613G* (42.8%), S694S (42.8%), and *P871L* (51.4%) (Table 12). The two most common mutations were the founder mutations common to women with an Ashkenazi Jewish descent. Among the subjects, 40 (29%) women had the 185delAG mutation and 16 (12%) had the 5382insC mutation. The other mutations found in this population are listed in Table 13.

Table 12. Most commonly found SNPs in sequenced population of familial breast cancer subjects (n=35)

BRCA1 SNP	Frequency	(%) of population
Q356R	8	22.86%
S2414S	12	34.29%
E1038G	14	40.00%
K1183R	14	40.00%
L771L	15	42.86%
S1436S	15	42.86%
S1613G	15	42.86%
S694S	15	42.86%
P871L	18	51.43%

Table 13. BRCA1 mutations found in population (n=138).

Mutation	Exon	Codon	Base change	A A change	Mutation type	Effect
R71G	5	71	A to G	Arg to Gly	M	Non-synonymous
R1347G	11	1347	A to G	Arg to Gly	UV	Non-synonymous
C61G	5	61	T to G	Cys to Gly	M	Non-synonymous
185delAG	2	23	delAG	stop 39	F	Truncation
E29X	3	29	G to T	Glu to Stop	N	Truncation
1294del40/V772A	11	392	del40	stop397	F	Truncation
1793delA	11	558	delA	stop571	F	Truncation
1996ins4	11	626	insTAGT	stop627	F	Truncation
2080delA	11	654	delA	stop700	F	Truncation
2594delC	11	825	delC	stop845	F	Truncation
2606delT	11	829	delT	stop845	F	Truncation
2634delC	11	839	delC	stop845	F	Truncation
2711delA	11	864	delA	stop893	F	Truncation
2800delAA	11	894	delAAGC	stop901	F	Truncation
3600del11	11	1161	del11	stop1163	F	Truncation
3819del5	11	1234	delGTAA	stop1241	F	Truncation
3875del4	11	1252	delGTCT	stop1262	F	Truncation
3878insT	11	1253	insT	stop1254	F	Truncation
3889delAG	11	1257	delAA	stop1265	F	Truncation
4184del4	11	1355	delTCAA	stop1364	F	Truncation
943ins10	11	275	ins10	stop275	F	Truncation
E1134X	11	1134	G to T	Glu to Stop	N	Truncation
G813X	11	813	G to T	Gly to Stop	N	Truncation
K1290X, L1564P	11	1290	A to T	lys to Stop	N	Truncation
K679X	11	679	A to T	lys to Stop	N	Truncation
Q1323X	11	1323	C to T	Gln to Stop	N	Truncation
Y978X	11	978	T to G	Tyr to Stop	N	Truncation
Q1395X	12	1395	C to T	Gln to Stop	N	Truncation
5055delG	16	1646	delG	stop1657	F	Truncation
5083del19	16	1655	del19	stop1670	F	Truncation
5382insC	20	1756	insC	stop1829	F	Truncation
E1817X	23	1817	G To T	Glu to Stop	N	Truncation
W1815X	23	1815	G to A	Trp to Stop	N	Truncation
R1835X	24	1835	C to T	Arg to Stop	N	Truncation
L752X	NR	11		leu to stop	N	truncation
Y101X	NR	7		Tyr to Stop	N	truncation
E143X	NR	7				truncation
C64Y	5	64	G to A	Cys to Tyr	M	synonymous
V772A	11	772	T to C	Val to Ala	UV	synonymous
L1564P	16	1564	T to C	Leu to Pro	UV	synonymous
C4446T	NR			Cys to Thr	UV	synonymous
L246V	NR	11	T to G	leu to Val	UV	synonymous
4794InsA	NR	15				unknown
5210delT	NR	18/19				unknown
Gint4T	NR					unknown
IVS20+1G>A						unknown
IVS4-1G>T						unknown
T>Gins59bp						unknown

The MBPC (mean breaks per cell) for women with the 185delAG, 5382insC, and other BRCA1 mutations was 0.36±0.44SD, 0.32±0.19SD, and 0.27±0.22SD, respectively. Subset analysis for women revealed that there was no significant association between mutation and MSA (mutagen sensitivity assay) (p=0.16; Table 14). Additionally, it should be noted that all C61G (n=5) subjects had high MBPC (mean breaks per cell) (mean=0.38, SD=0.19), but no statistical differences were found when compared to other mutation carriers (mean=0.29, SD=0.29; p=0.47) (Table 15).

Table 14. Relationship between Mutagen Sensitivity and specific *BRCA1* mutation after removing related individuals from analysis. *Adjusted for age.

	Low	High	OR	95% CI	OR*	95% CI	Wald $\chi 2$	p
Other	34	35	1.00					
187delAG	7	21	2.90	1.10 - 7.70	1.01	0.93 - 1.10		
5382insC	4	12	*2.90*	*0.90 - 9.90*	*1.05*	*0.93 - 1.17*	*1.95*	*0.16*

Table 15. DNA repair capacity in C61G mutation carriers.

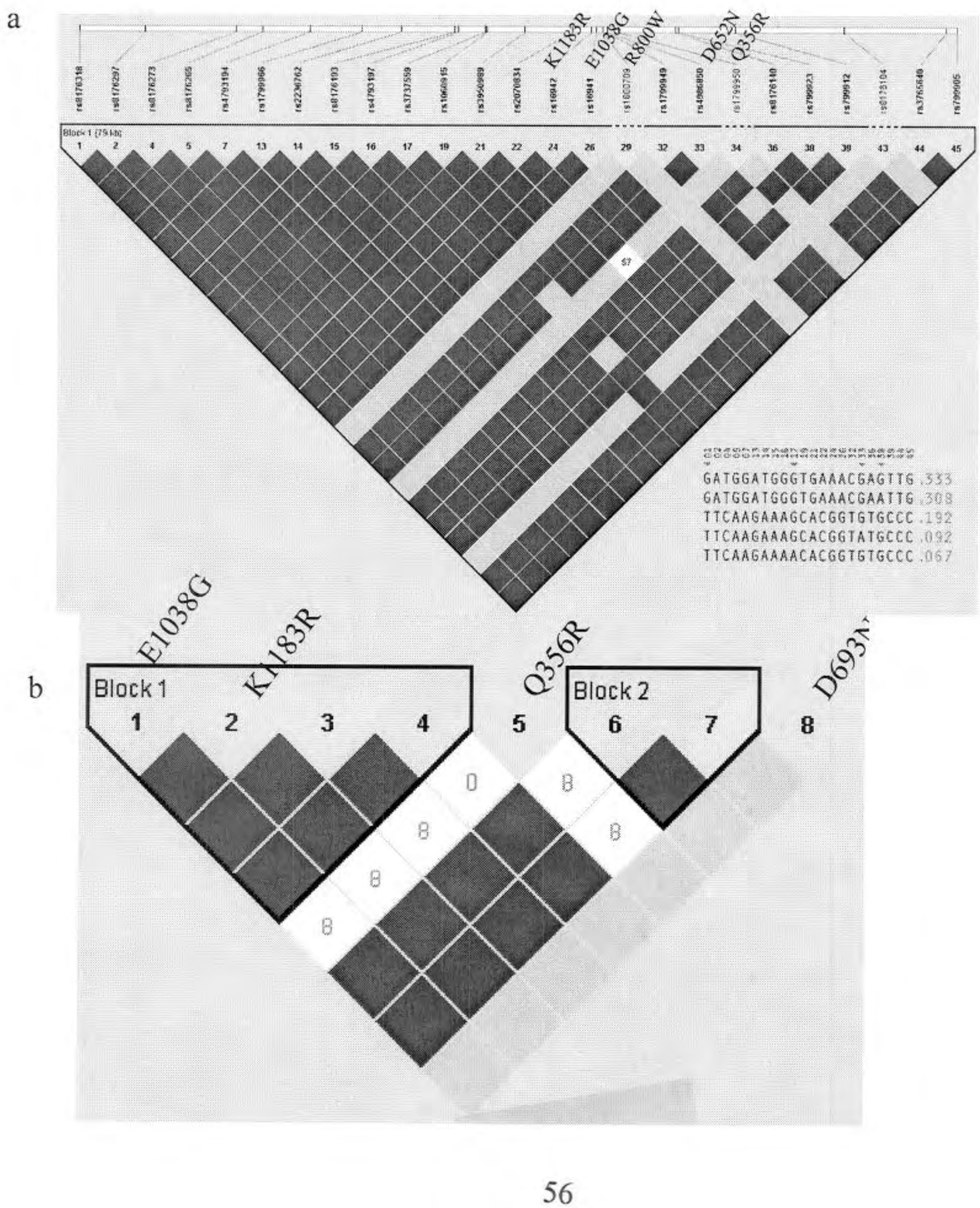
LCC ID	MBPC	Age	Breast Cancer type
15857	0.23	38	DCIS, Infiltrating ductal carcinoma, metasttic carcinoma
14471	0.72	30	DCIS, Infiltrating ductal carcinoma, metasttic carcinoma, bilateral breast cancer
14462	0.3	31	None
14460	0.3	27	None
14467	0.36	50	Infiltrating ductal carcinoma

BRCA1 Genotype and Haplotype-Phenotype correlations

Myriad sequencing data was used to determine SNPs to assess in the population (n=138). Haplotypes were also constructed and Haploview (Haploview 3.32, Boston, MA; Barret JC 2005) identified tagSNPs in the general Caucasian population that were also used for individual genotyping analysis. Four tags are found in the coding regions and the other 3 were found in

promoter or non-coding regions. Figure 14 displays Haploview results for the general population (Figure 15a) and subjects with prior *BRCA1* sequencing (Figure 15b). Haplotypes from the studied population deviated slightly from the general Caucasian population. However, they both agreed on the 3 tag SNPs identified. The *E1038G*, *Q356R*, and *D693N* SNPs were used for genotyping and haplotyping analysis.

Figure 15. a) Haploview results for the general population and b) subjects with prior *BRCA1* sequencing.



In the *E1038G* polymorphism, data in the present study were available for 110 women. A χ^2 test of observed versus expected genotype frequencies for the *E1038G* polymorphism among subjects followed Hardy–Weinberg equilibrium (p=0.70) (Table 16). After adjusting for age, it was found that those with at least one variant C allele were *not* more likely than women with the TT genotype to have high MBPC (mean breaks per cell) (OR:1.08 95% CI:0.47-2.46) and neither homozygosity nor heterozygosity for the C variant was associated with increased DNA repair efficiency (Table 17). In addition, there were no significant differences between genotypes when $\chi2$ analysis was done ($\chi2$=0.55, p =0.46).

Table 16. *BRCA1 E1038G* $\chi2$ analysis and test for Hardy-Weinberg.

Genotype	Observed	Expected	$\chi2$	p
TT	0.39	0.39		
CT	0.45	0.47		
CC	0.15	0.14	0.15	0.70

Table 17. Relationship between Mutagen Sensitivity and *BRCA1 E1038G* polymorphism in unrelated individuals.

Genotype	Low	High	OR	95% CI	OR*	95% CI	Wald $\chi2$	p
TT	20	23	1.00		1.00			
CT	18	32	1.13	0.47 - 2.73	1.08	0.45 - 2.64		
CC	6	11	1.21	0.37 - 3.99	1.07	0.32 - 3.60	0.55	0.46
C allele	24	43	1.60	0.70 - 3.40	1.08	0.47 - 2.47	0.91	0.34

***Adjusted for age**

In the *D693N* polymorphism, data in the present study were available for 110 women. A χ^2 test of observed versus expected genotype frequencies for the *D693N* polymorphism among subjects followed Hardy–Weinberg equilibrium (P = 0.70) (Table 18). When adjusted for age, subjects with T allele were *not* more likely than women with the CC genotype to have high

MBPC (mean breaks per cell) (OR: 6.03, 95%CI: 0.69-52.02) (Table 19). In addition, there was only marginally significant differences between genotypes when $\chi 2$ analysis was done ($\chi 2$=0.10).

Table 18. *BRCA1 D693N* $\chi 2$ analysis and test for Hardy-Weinberg.

Genotype	Observed	Expected	$\chi 2$	*p*
CC	0.9	0.9		
CT	0.092	0.097		
TT	0	0.003	0.25	0.61

Table 19. Relationship between Mutagen Sensitivity and *BRCA1 D693N* polymorphism in unrelated individuals.

Genotype	Low	High	OR	95% CI		OR*	95% CI		Wald $\chi 2$	*p*
CC	41	57	1.00			1.00				
CT	1	9	5.98	0.69	51.06	6.03	0.69	52.02	2.67	*0.10*

***Adjusted for age.**

In the *Q356R* polymorphism, data in the present study were available for 110 women. A χ^2 test of observed versus expected genotype frequencies for the *Q356R* polymorphism among subjects followed Hardy–Weinberg equilibrium ($P = 0.29$) (Table 20). After adjusting for age, subjects with the C variant allele were *not* more likely than subjects with the TT genotype to have high MBPC (mean breaks per cell) (OR: 0.57, 95%CI: 0.20-1.60) (Table 21). In addition, there were no significant differences between genotypes when $\chi 2$ analysis was done ($\chi 2$=0.28).

Table 20. *BRCA1 Q356R* $\chi 2$ analysis and test for Hardy-Weinberg.

Genotype	Observed	Expected	$\chi 2$	*p*
TT	0.81	0.81		
CT	0.18	0.18		
CC	0	0.01	1.14	0.29

Table 21. Relationship between Mutagen Sensitivity and *BRCA1 Q356R* polymorphism in unrelated individuals.

Genotype	Low	High	OR	95% CI	OR*	95% CI	Wald $\chi 2$	*p*
TT	32	56	1.00		1.00			
CT	11	9	0.54	0.19 - 1.50	0.57	0.20 - 1.60	1.15	0.28

***Adjusted for age.**

Gene-gene interactions were examined for *BRCA1* mutations, *BRCA1* SNPs and *Rad51* SNPs. Although the numbers of subjects become small, there was a borderline association for the *E1038G* SNP in *185delAG* mutation carriers. In the 185delAG analysis, significant differences between genotypes were found with the *E1038G* polymorphism (Table 22). The OR for the TT, CT, and CC alleles were 1.0, 10.70 (95%CI: 0.70, 158.50), and 4.0 (95%CI: 0.20-75.70), respectively. For the CC and CT genotypes, the OR was 8.0 (95%CI: 0.60-106.90). For *Rad51*, the OR was also elevated (OR= 3.70, 95% CI: 0.40-36.60), but was not statistically significant (p=0.21). For the *BRCA1 5382insC* mutation, the analysis revealed a decreased risk for high MBPC (mean breaks per cell) in both the *Q356R* and *D693N* polymorphism (OR=0.30, 95%CI: 0.01-6.40 and OR=0.40 95% CI: 0.10-8.10, respectively), but results were not statistically significant (p=0.29 and 0.73, respectively). For *Rad51*, the OR was, again, elevated (OR=2.0 95%CI: 0.10-26.70), but was not statistically significant (p=0.42).

Table 22. Association between genotype and mutagen sensitivity in 185delAG mutation carriers (n=28)

		Low	High	OR	95% CI	Fisher's p
E1038G	TT	2	1	1.00		
	CT	3	16	10.70	0.70 - 158.50	
	CC	2	4	4.00	0.20 - 75.70	*0.04*
	CC and CT	5	20	8.00	0.60 - 106.90	*0.14*
D693N	CC	6	18	1.00		
	CT	0	3	1.00	0.10 - 11.50	0.45
Q356R	TT	5	18	1.00		
	CT	1	3	0.80	0.10 - 9.90	0.45
Rad51	GG	6	13	1.00		
	CG/CC	1	8	3.70	0.40 - 36.60	0.21

Table 23. Association between genotype and mutagen sensitivity in 5382insC mutation carriers (n=15).

		Low	High	OR	95% CI	Fisher's p
E1038G	TT	3	5	1.00		
	CT/CC	1	6	1.70	0.30 - 10.30	0.28
D693N	CC	4	10	1.00		
	CT	0	1	0.40	0.01 - 8.10	0.73
Q356R	TT	3	10	1.00		
	CT	1	0	0.30	0.01 - 6.40	0.29
Rad51	GG	3	6	1.00		
	CG/CC	1	4	2.00	0.10 - 26.70	0.42

Haplotype results in high risk subjects

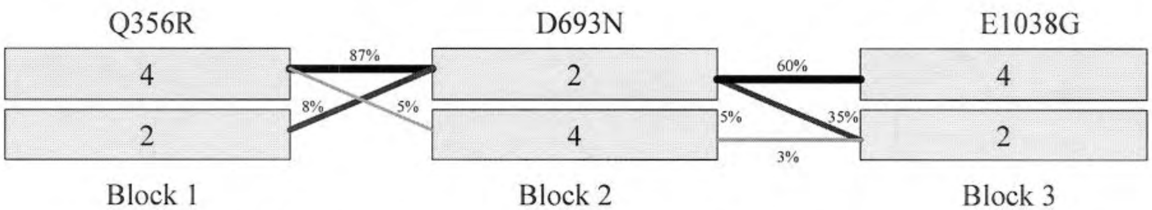
Haplotypes were constructed using the data described above Haploview and Phase Software (as described in methods). Phasing analysis revealed a total of 5 haplotypes and their total and allele frequencies are listed in Table 24a which shows that frequencies ranged from 1.9% to 54.2%. Haplotype pair frequencies are also listed in Table 24b and it showed frequencies ranging from 0.7% to 30.9%. A high density SNP map across the *BRCA1* locus was assembled to determine LD block and haplotype structure (Figure 16).

Table 24. a) BRCA1 Haplotypes and frequencies. b) Haplotype pair frequencies. The numbers for each SNP correspond to the nucleotide at that position (1=A, 2=C, 3=G, 4=T). 1st position= *Q356R*; 2nd position=*D693N*; 3^rd^ position = *E1038G*

	Haplotypes	Freq.	Freq. (%)
1	424	148	0.542
2	422	94	0.329
3	442	13	0.044
4	224	21	0.064
5	222	2	0.019

Pairs	Freq.	Freq. (%)
11	43	0.309
12	43	0.309
13	9	0.065
14	10	0.065
22	18	0.072
23	3	0.007
24	10	0.022
25	2	0.014
34	1	0.007

Figure 16. *BRCA1* LD blocks and haplotypes patterns. The lines between the blocks link haplotypes that are transmitted with greater than 2.5% frequency across blocks.



Analysis to determine if a haplotypes was associated with a specific mutation was done and although $\chi 2$ analysis revealed significant differences between haplotypes in relation to mutations (p=0.05), no association was found between a haplotypes and a specific mutation (Table 25). The analysis to determine if *BRCA1* haplotypes and mutagen sensitivity were related was done and, again, no association was found (p=0.17; Table 26). ORs for the 424, 422, 224, 442, and 222 haplotypes were 1.0, 1.1 (95%CI: 0.58-1.95), 0.5 (95%CI: 0.19-1.42), 5.9 (95%CI: 0.75-47.9), and 0.7 (95%CI: 0.04-10.69), respectively.

Table 25. Association between haplotype and *BRCA1* mutation.

	185delAG	5382insC	other	OR*	95%CI	$\chi 2$	p
424	21	23	75	1			
422	28	7	36	0.89	0.47-1.71		
224	4	1	13	0.55	0.19-1.56		
442	3	1	6	6.17	0.72-52.4		
222	0	0	2	1.05	0.06-17.75	15.53	0.049

Table 26. Association between haplotype and mutagen sensitivity.

	Low	High	OR	95%CI	$\chi 2$	Fisher's p
424	47	72	1.0			
422	27	44	1.06	0.58-1.95		
224	10	8	0.52	0.19-1.42		
442	1	9	5.88	0.72-47.9		
222	1	1	0.65	0.04-10.69	5.75	0.17

Aim 4: To identify single nucleotide polymorphisms (SNPs) or haplotypes in *BRCA1* and *Rad51* associated with increased risk to sporadic breast cancer.

Hypothesis 4: SNPs and haplotypes associated with a DNA repair phenotype in hereditary breast cancer will modify risk in cases versus controls.

Rad51 5'UTR 135G>C genotyping results in case-control study

The characteristics of the WEB Study participants are shown in Table 27. Both cases and controls were predominantly Caucasian (>90%). Blinded quality control duplicates were 100% concordant for the *Rad51* genotype.

Table 27. The characteristics of the WEB Study participants (n=2994).

Characteristics	Premenopausal		Postmenopausal	
	Cases	Controls	Cases	Controls
N	301	556	777	1360
		Mean		
Age, years	44.8	44.1	63.2	63.2
(SD)	4.5	4.6	8.4	8.8
Education, years	13.9	14.3	13.4	13
(SD)	2.3	2.2	2.6	2.3
Body mass index	27.2	27.5	29	28.5
(SD)	7.1	6.6	6.1	6.1
Age at menarche, years	12.6	12.6	12.5	12.7
(SD)	1.6	1.6	1.6	1.7
Parity	1.9	2	2.5	3
(SD)	1.3	1.3	1.8	1.9
Age at first birth, years	25	25.9	23.9	23.6
(SD)	5.1	4.8	4.7	4.3
Age at Menopause	-	-	48.2	47.3
(SD)			5.4	6.3
Caucasian (%)	94.3	94.6	93.9	90.4
African American (%)	4.9	3.8	4.8	8.5
History of benign breast disease (%)	35.9	22.1	34	22.7
Family history of breast cancer (%)	18.3	9.9	18.9	13.5
Current smoker (%)	38.4	30.8	11.3	14.8

Rad51 genotypes in controls were in Hardy-Weinberg equilibrium for both whites (n=1475 GG, n=256 CG, n=15 CC, p=0.30), but were different when non-whites were included (p=0.07) (Table 28).

Table 28. ***Rad51*** **5'UTR 135G>C test for Hardy-Weinberg among cases and controls.**

	Observed Genotype						Expected			Hardy-Weinberg Equilibrium	
Rad51 rs1801320	**CC**	**CG**	**GG**	**ND**	**freq p**	**freq q**	**p2**	**2pq**	**q2**	**$\chi2$**	***p*-value**
Controls	23	307	1586	29	0.09	0.91	16	320	1579	3.39	0.07
Cases	14	186	878	21	0.1	0.9	11	193	875	1.33	0.25
Controls (Whites only)	15	256	1475	27	0.08	0.92	12	263	1472	1.1	0.3
Cases (Whites only)	9	156	830	18	0.09	0.91	8	159	829	0.31	0.58

Subsequently, a comparison was made between whites and non-whites and it was found that frequencies between them were significantly different (Table 29). Non-whites were almost 8 times more likely to have the CC genotype (OR: 7.9, 95%CI=3.9-15.7; p<0.001).

Table 29. Allele frequencies in Whites compared to Non-whites.

	Whites	Non-Whites	OR	95% CI
GG	2305	159	1.00	
CG	412	81	2.9	2.1-3.8
CC	24	13	7.9	3.9-15.7
C allele	436	94	3.1	2.4-4.1

*$\chi2$=87.32 p<0.001

Characteristics of the study sample were stratified by menopausal status and genotypes (Table 30). Two-sided comparisons of means between the cases and controls were computed by T-test for continuous variables and by $\chi2$ test for categorical variables and, although, statistical differences were not found between cases and control, characteristics were controlled for in the risk assessment of breast cancer by *Rad51* genotype (Table 31). Because there were few differences in relationships for those with CC and CG genotypes, these categories were collapsed and the associations between genotype and risk were evaluated. Logistic regression indicated that there was no significant difference in genotype distribution between cases and

controls when stratified by menopausal status and after adjustment for confounding variables (OR=0.87, 95% CI= 0.57-1.31 for premenopausal and OR=1.11, 95% CI= 0.86-1.44 for postmenopausal, respectively) (Table31).

Table 30. Characteristics of Study Sample by Case-Control Status and Genotypes, WEB Study

Characteristics*	Pre-menopausal				Post-menopausal			
	Cases		Controls		Cases		Controls	
	GG	CC+CG	GG	CC+CG	GG	CC+CG	GG	CC+CG
N	251	50	456	100	627	150	1130	230
Age(years)	44.9 (4.7)	44.6 (3.9)	44.1 (4.7)	44.1 (4.6)	63.1 (8.5)	63.6 (8.5)	63.1 (8.8)	63.8 (9.0)
Education (years)	13.9 (2.3)	13.8 (2.1)	14.3 (2.2)	14.1 (2.1)	13.3 (2.7)	13.4 (2.6)	13.0 (2.3)	12.8 (2.3)
Race (% of whites)	94	86	96.3	86	94.7	81.3	91.7	80.4
Body Mass Index	27.3 (6.9)	27.0 (6.8)	27.1 (6.4)	28.8 (7.7)	28.8 (5.9)	29.3 (6.5)	28.4 (6.1)	29.0 (5.9)
Age at menarche (years)	12.5 (1.6)	13.1 (1.6)	12.6 (1.5)	12.6 (1.9)	12.6 (1.6)	12.2 (1.7)	12.7 (1.7)	12.9 (1.6)
Age at menopause	/	/	/	/	48.2 (5.4)	48.7 (5.1)	47.3 (6.2)	47.6 (6.8)
Number of births	1.9 (1.3)	1.8 (1.1)	2.0 (1.2)	1.8 (1.3)	2.5 (1.8)	2.7 (1.9)	3.0 (1.9)	3.1 (1.8)
Percentage of women without child (%)	17.1	16	14.7	19	18.2	15.3	10.1	10.4
First-degree relative with breast cancer (% of Yes)	19.1	14	9	14	18.2	22	13.5	13
Previous benign breast disease (% of Yes)	34.7	42	22.4	20	34	31.3	22.7	22.2

Values for continuous variables are mean (SD); for categorical variables (race, first-degree relative with breast cancer, history of benign breast disease) values are for percentages among either the cases or controls. Two-sided comparisons of means between the cases and controls were computed by T-test for continuous and by χ square test for categorical variables.

Table 31. Risk of Breast Cancer by *Rad51* Genotype

RAD51	Cases	Controls	Crude OR	95% CI	Adjusted OR*	95% CI
			Pre-menopausal			
GG	251	456	1.00		1.00	
CG+CC	50	100	0.91	0.63 - 1.32	0.83	0.56 - 1.23
			Post-menopausal			
GG	627	1130	1.00		1.00	
CG+CC	150	230	1.18	1.18 - 1.48	1.19	0.93 - 1.51

*Odds ratios and 95% confidence intervals adjusted for age, education, race, BMI, age at first birth, age at menarche, age at menopause (for post-menopausal women only), number of births, first-degree relative with breast cancer, and previous benign breast disease.

Key Research Accomplishments and Findings

Aim 1

- Mutagen sensitivity assay validation well correlated in peripheral blood lymphocytes and EBV-immortalized cell lines and there is a good relationship in mutagen sensitivity between EBV-immortalized lymphocytes and peripheral blood lymphocytes
- Cancer status does not affect mutagen sensitivity

Aim 2

- There are a range of mutagen sensitivity responses in subject population
- Affected subjects are more likely to have higher mean breaks per cell compared to unaffected subjects
    - Affecteds have a 2-3 fold chance of having higher mean breaks per cell compared to unaffected
    - Having high mean breaks per cell seems to be a characteristic of cancer-affected individuals

Aim 3

- Subjects with the 5382insC and 185delAG mutation were not more likely to have higher MBPC compared to other mutations
- E1038G polymorphism not associated with MBPC in this population
- D693N polymorphism may be associated with high MBPC, OR=6.03 (0.69-52.02) (p=0.10)
- Q356R polymorphism not associated with MBPC in this population
- In 185del AG mutation carriers, E1038G polymorphism maybe associated with high MBPC OR=8.0 (0.60-106.90), p=0.14
- In 5382insC mutation carriers, D693N and Q356R polymorphisms associated with low MBPC, OR=0.40 (0.01-8.10) and 0.30 (0.01-6.40), respectively
- BRCA1 haplotypes may be associated with specific mutations (p=0.049)
- BRCA1 haplotypes may be associated with MBPC (p=0.17)
- Rad51 sequencing was performed, no polymorphisms found (with >5% frequency)
- Rad51 5'UTR 135G>C, CC/CT (variant allele) OR=2.50 (1.02-6.13), p=0.03
- In 185delAG and 5382insC mutation carriers, subjects with CT and CC genotypes associated with high MBPC, OR=3.70 (0.40-36.60) and 2.0 (0.10-26.70), respectively

Aim 4

- Rad51 5'UTR 135G>C polymorphism not associated with sporadic breast cancer risk
    - But it was not examined in non-whites
    - And it was not assessed by non-genetic factors

Reportable Outcomes

1. **Manuscript to be submitted to *Cancer Research:***

"Rad51 5'UTR 135G>C polymorphism associated with DNA repair capacity in Hereditary Breast Cancer Patients"
Luisel Ricks-Santi, Jo Freudenheim, Francoise Seillier-Moiseiwitsch, Claudine Isaacs, Marc Schwartz, Ramona Dumitrescu , Catalin Marian, Jing Nie, Dominica Vito, Maurizio Trevisan, Stephen Edge, Peter G. Shields

2. **Presented at 2006 Annual American Association for Cancer Research**

"Induced chromosomal aberrations, genetics, and pathology in hereditary breast cancer"
Luisel Ricks-Santi, Camille Jasper, Marc Schwartz, Claudine Isaacs, Peter G. Shields

3. **Awards received based on abstract:**

American Association for Cancer Research Travel Fellowship

4. **Degrees obtained supported by this award:**

PhD Tumor Biology- Dissertation entitled "DNA repair genotypes and associated phenotypes"

5. **Research opportunities applied for based on experience/training supported by this award**

NCI Cancer Prevention Fellowship
NCI Department of Cancer Epidemiology and Genetics

Conclusions

In this study, we first validated the MSA (mutagen sensitivity assay) in EBV-immortalized lymphocytes. We showed that the MSA (mutagen sensitivity assay) in EBV-immortalized lymphoblasts is reproducible and representative of the MSA (mutagen sensitivity assay) performed on freshly cultured blood. Then we examined the relationship between the MSA (mutagen sensitivity assay) and specific SNPs and haplotypes in *BRCA1* and SNPs in *Rad51*. The *Rad51 5'UTR 135G>C* SNP was statistically associated with increased MBPC (mean breaks per cell) in high risk families, but was not associated with risk in the WEB case-control study. There was also increased risk of MBPC (mean breaks per cell) with the *BRCA1 D693N* genotype, but was not statistically significant (p=0.10). There was no association between the *BRCA1 Q356R* and *E1038G* genotypes or *BRCA1* haplotypes and MSA (mutagen sensitivity assay). When stratified by *185delAG* or *5382insC* mutations, an elevated OR with the *E1038G* polymorphism and decreased ORs were found with the *D693N* and *Q356R* genotypes indicating them may be a modifier of *BRCA1* risk. Elevated ORs were also found in *Rad51* in 185delAG and 5382insC mutation carriers, but was not statistically significant. This suggests that the *Rad51 5'UTR 135G>C* SNP, *BRCA1 Q356R, D693N,* and *E1038G* genotypes may be modifying the penetrance of *BRCA1* mutations in high risk families.

The Mutagen Sensitivity Assay

The first step in this project was validation of the mutagen sensitivity assay. Given that the use of fresh peripheral blood, cryopreserved blood, and EBV-immortalized cell lines lead to the MSA (mutagen sensitivity assay) study of different lymphocyte subpopulations (B-cell

lymphocytes vs. T-cell lymphocytes), it has not been established that these will provide similar results and risk estimates because the different cell types might affect the predictive capability for the MSA (mutagen sensitivity assay) assay. Despite the fact that studies have compared different mutagen sensitivity assays in the same population, the results have shown that the different assays could be measuring different DNA repair capacities (26, 158). For example, Trenz et al. used both the micronucleus and COMET assays in fresh blood and immortalized cell lines to measure radio-sensitivity in BRCA1 and BRCA2 mutation carriers and healthy controls; initially, a relationship between mutation and mutagen sensitivity was observed using the micronucleus assay and no relationship was seen using the COMET assay (158), but later, they showed that lymphoblastoid cell lines with the same BRCA1 mutations did not show the same radio-sensitivity (159-161). A comparison between the micronucleus assay and the MSA (mutagen sensitivity assay) was also done, and again, no correlation between the assays was found (26). An assessment of micronuclei formation following radiation exposure of lymphocytes where a comparison of fresh blood and cryopreserved was done but comparisons showed a poor correlation (146).There is even limited data for other types of assays assessing mutagen sensitivity in EBV-immortalized lymphocytes, which show that immortalized lymphocytes are a good model for the assessment of DNA repair capacity (26, 136, 145, 158-164). However, none of these studies validate or directly compare the MSA (mutagen sensitivity assay) for freshly cultured cells and concordant EBV immortalized cells in the same study subjects, it was unknown if prior results are due to differences in study subject or assay results. This study is the first report that we know of concordant peripheral blood lymphocytes and EBV-immortalized lymphoblasts comparison using the MSA (mutagen sensitivity assay). Baeyens et

al. compared DNA repair capacity in concordant fresh PBL and EBV-immortalized cell lines using the micronucleus assay, although not the MSA (mutagen sensitivity assay), in both breast cancer patients and healthy donors, a direct correlation between values was not calculated (165). They also showed that although no differences in spontaneous micronuclei were found in EBV-immortalized cell lines and fresh blood, results did not show elevated micronuclei in EBV-immortalized cell lines from breast cancer patients with increased micronuclei in fresh blood. While the mutagen sensitivity assay has shown consistent results in breast cancer risk studies, the micronucleus (MN) assay and COMET assay do not, even when compared to each other in concordant samples. Therefore, the MN and COMET assays may not be the appropriate methods to study DNA repair capacity and breast cancer risk. In this study it was shown that EBV-immortalized cell lines, although of B-lymphocyte origin, have similar quantitative results to freshly cultured whole blood, which yields mostly T-lymphocytes ($p=0.11$). The results reported herein show that there were strong correlations between concordant samples for both replicate analysis of the same cultures and cultures thawed and established on different days. The coefficient of variation 46.03% and this is acceptable because variability exists between individuals (inter-individual variation). There is even variation within individuals on any given day, but CV is expected to be a lot lower than inter-individual variation. We had CVs ranging from 0.0%-28.28% within individual samples.

Compared with previous results for γ-radiation-induced MSA (mutagen sensitivity assay), the MBPC (mean breaks per cell) for MSA (mutagen sensitivity assay) from this study was relatively lower. Differences could arise from variation in the study population, radiation

dose, post-radiation incubation time, experimental handling, and slide-reading criteria. Table 1 in Chapter 2 listed MSA (mutagen sensitivity assay) study characteristics, such as radiation dosage, post-incubation irradiation time, etc. The use of 1Gy γ-irradiation was relatively lower compared to other studies (1.5Gy), as was post-irradiation incubation time (4hours vs. 0.5-1.5 hours) (20-22, 108, 109, 118, 119, 122). It is possible that the radiation dose used in the present study may have been low, but differences were between affected and unaffected individuals were detected. While others have successfully detected differences with as much as 4Gy, dose response evaluations showed 100% cell death in the current population with the use of 2Gy. This could be a result of radio-sensitivity due to germline BRCA1 mutations. Also, in this study, only frank chromatid breaks were counted and all gaps were excluded, whereas some of the others also counted gaps. Also, for this study, γ-radiation for the MSA (mutagen sensitivity assay) was chosen as the mutagen, as has been done for other breast cancer studies MSA (mutagen sensitivity assay) studies (22, 24, 166), and because γ-radiation is a direct DNA-damaging agent that is not dependent on cell penetration or metabolism. Provided that the cytogeneticist maintains the criteria and is blinded, results would be valid. In this study, cell culture, harvesting, cell preparation, staining, and scoring were done by a single cytogeneticist (LRS) for the entire study population.

When the relationship between the MSA (mutagen sensitivity assay) and cancer status was assessed, the data also shows that affected subjects had higher MBPC (mean breaks per cell) than unaffected subjects (OR=3.2 95%CI: 1.5-6.7; $\chi^2 = 7.1$, p=0.0077). Almost 1/3 of study participants were related, and because family members share genetics and mutagen sensitivity, it

was important to do separate analysis of unrelated subjects. For this study, only probands, the first individual in the family recruited in the study, were included. When related individuals were removed from the analysis, the median in the unaffected subjects changed from 0.18 to 0.22 MBPC (mean breaks per cell). Consequently, the association between mutagen sensitivity and cancer status decreased to borderline significant (OR=2.0, 95% CI: 0.9-4.6; $\chi^2 = 3.02$, p=0.10) from significant (OR=3.02, 95%CI: 1.5-6.7). This may be due to the decrease in sample size and decrease in statistical power. However, results were still consistent with other studies showing decreased DNA repair capacity in breast cancer cases compared to controls (20-22, 24, 25, 117-122). The MSA (mutagen sensitivity assay) has also been shown to be a heritable trait and in a twin study, Wu and colleagues identified a high concordance among monozygotic twins, and a lower concordance among dizygotic twins (115). The MSA (mutagen sensitivity assay) also has previously been shown to be a breast cancer risk factor in high-risk families (20-22, 108-116). For this second reason, it was important to do separate analysis excluding related individuals.

Of subjects were currently being treated or recently treated, it is conceivable that the MSA (mutagen sensitivity assay) might be affected due to changes in blood lymphocytes. For the MSA (mutagen sensitivity assay), MBPC (mean breaks per cell) is determined by the count of chromosomal breaks in 50-100 metaphases. For some subjects, less than the required amount of metaphases could be obtained. Of the 138 subjects, 43 (31%) did not have 50 metaphases. Specifically 41 (73%) of the unaffecteds and 54 (66%) of the affecteds had 50 metaphases. However, subjects with less than 50 metaphases were not more likely to be affected ($\chi2$=0.22, p=0.64) or have high MBPC (mean breaks per cell) ($\chi2$=0.71, p=0.39). Additionally,

dichotomization of values into high and low MBPC (mean breaks per cell) helps to deal with extremes and outliers, if any. It is conceivable that these subjects were recently treated prior to blood collection.

Genetic Trait Penetrance and Familial/Sporadic Breast Cancer Risk

Inherited *BRCA1* and *BRCA2* mutations strongly predispose individuals to breast and ovarian cancer; however, having the mutation does not confer 100% predisposition and/or mortality. A meta-analysis of 22 population based studies of *BRCA1* penetrance revealed that risks vary in both *185delAG* and *5382insC* mutation carriers (167). The hypothesis that the variable penetrance might be due to other lower penetrant susceptibilities has been previously studied. An initiative to identify BRCA1 genetic modifiers has led to the development of the Consortium of Investigators of Modifiers of *BRCA1* and *BRCA2* (CIMBA). It has been proposed that genes in specific pathways might be responsible for BRCA1 risk modification, pathways of steroidigenesis, DNA repair, and carcinogen detoxification. For example, the CAG repeat length polymorphism in the androgen receptor (AR) gene has been examined in *BRCA1* and *BRCA2* mutation carriers, but there have been inconsistent findings (168-171). Also, *AIB* (Amplified in Breast caner gene) has been studied in these high-risk individuals, and again, contradictory results have been found (172-175). The prohibitin (PHB) gene has also been found to increase the risk of breast cancer in patients with the 5382insC (176). *Rad51* has also been proposed as a risk modifier in BRCA1 mutation carriers, and the association studies will be discussed in the *Rad51* and DNA Repair Section of this discussion.

Studies of genetic susceptibilities and breast cancer risk in the general population are also actively being studied. There are conflicting data and few single SNP have been shown to consistently increase breast cancer risk (Table 1). For example, in the *HER2/neu* gene, the 655V variant has been inconsistently associated with breast cancer risk. In the Nurse's Health Study, this variant was associated with breast cancer risk (177), but other smaller studies failed to confirm positive results (178-180). The same was found for *VDR* (181-183) and *PGR* (184-186). However, given the large number of SNPs in the genome, it is likely that many positive associations are false positives. That is why the use of phenotypic assays along with genotype analyses are important. In the *HER2/neu* study, Paputti et al performed *in vitro* analyses and measured receptor amplification. Although, not statistically significant, this study showed both allelic and protein differences between breast tumors and control samples (180). The results of this assay could be used for the development of an *a priori* hypothesis for the establishment of *HER2* polymorphisms and breast cancer risk.

Table 1. Breast Cancer risk association studies.

Gene	Polymorphism	Association	Reference
CYP19	TTTA(n)	+	(196)
CYP19	haplotypes	-	(199)
CYP19	TTTA(n)	+	(201)
erbB2/HER2	655Val	+	(177)
erbB2/HER2	655Val	-	(178)
erbB2/HER2	655Val	-	(179)
FTHFD	intronic	+	(180)
HSD17B1	Ser312Val	-	(200)
MDM2	-309	-	(197)
MTHFD1	134K	+	(198)
PGR	331Ala	+	(186)
PGR	331Ala	-	(184)
PGR	331Ala	+	(185)
VDR	*FOK1* variant	+	(182)
VDR	*FOK1* variant	-	(183)
VDR	*FOK1* variant	+	(183)

BRCA1 and DNA Repair

Many studies have proven that loss of BRCA1 proteins results in defective DNA damage repair and genetic instability; this suggests that BRCA1 plays a major role in DNA repair. Specifically, BRCA1 has been shown to "sense" DNA damage and is involved in checkpoint control of the cell cycle (57-63). Genome integrity maintenance is critical for the survival of all living organisms and when DNA is altered or damaged, cells undergo apoptosis or cell arrest so the lesion can be repaired. Ultimately, deficiencies in BRCA1 and thus, DNA repair mechanisms, can give rise to cancer resulting from unrepaired DNA lesions, which result in DNA mutations. Consequently, mutations may cause the altered functions of cells resulting in tumorigenesis. Because the prevalence of BRCA1 mutations in the general population is low, limited conclusions can be drawn from genotype/phenotype studies. Even when an association is found, there is evidence that cancer risk can be modified by additional factors. The structure of

BRCA1 gives us a lot of information about its function. Specifically, the structure of BRCA1 reveals several domains that interact with a variety of proteins that function in the ubiquination pathway, as well as the DNA repair pathway. Particularly, the BRCA1 protein contains a RING finger domain (located at amino acids 24-64) (71) which are responsible for heterodimerization with BARD1 (BRCA1-associated RING domain protein 1). The BRCA1-BARD1 heterodimer contains significant ubiquitin ligase activity and can be disrupted by mutations in the ring finger domain of BRCA1 (73, 74). Constructs containing the deleterious C61G *BRCA1* mutation and other mutations in the BRCA1 ring finger domain were shown to completely abolish Ubiquitin ligase activity (187). Specifically, The C61G mutations seemed to alter one of the Zn+2 binding ligand sites in the RING finger motif resulting in increased susceptibility to proteolysis (73, 187) because of its inability to dimerize with itself or with BARD1. The C61G *BRCA1* mutation is one of the BRCA1 alteration most commonly found in Polish families (188) It is a missense mutation found in exon 5 of the *BRCA1* gene and was previously reported in various other ethnic groups, however, the highest incidence is found in Poland, being found in 15-20% of Polish Breast and Ovarian Cancer families (188-191).

The targets for BRCA1/BARD1 ubiquination still remain unknown, however, BRCA1 is known to associate with the holoenzyme of RNA polymerase II, which is ubiquinated and degraded after DNA damage. So it has been proposed that when the transcription machinery approaches the site of DNA damage, BRCA1-BARD1 mediated ubiquination of the RNA polymerase II holoenzyme occurs. So if BRCA1 is defective in ubiquination of the holoenzyme,

as a result of a mutation of the gene, BRCA1, also, cannot signal to the cell that DNA repair must take place, thus making it DNA repair deficient.

In the current study, it was found that all women carrying the C61G mutation had high mean breaks per cell, as determined by the median mean breaks per cell of the subjects without cancer in the study population. These women were relatively young (age range= 27-50), and three, aged 30, 33, and 50, had been diagnosed with advanced disease. The other two, aged 27 and 31, probably have a high likelihood of developing breast cancer at a young age, as well, and it is only a matter of time before they do. Although two of the five subjects were related (14462 and 14471), the impact of the mutation on DNA repair remains evident in all the subjects. DNA repair capacity as a heritable trait is evidenced by having two related individuals in the analysis with similar and identical MBPC (mean breaks per cell) values.

BRCA1 sequencing data was also available for subjects who were not from families with Ashkenazi Jewish founder mutations *5382insC* and *185delAG* which revealed data to identify genotypes and haplotypes relevant in this population. The most frequently found functional polymorphisms in those subjects were *P871L, E1038G, L771L, S1613G, K1183R,* and *S694S*. This information was used for the selection of SNPS to assess in this study and provided information for haplotype analysis and for the identification of tagging SNPs for *BRCA1*: *Q356R, D693N,* and *E1038G*. While results were null for the *BRCA1 E1038G* and *Q356R* genotypes, the data indicates that there is a large elevated risk of high MBPC (mean breaks per cell) for the *D693N* genotype (OR=6.03, 95%CI: 0.69-52.02), but was only marginally significant ($p=0.10$).

When stratified by mutation, sample size became small, but differential ORs could be seen. For example, the BRCA1 *E1038G CT* genotype was associated with high MBPC (mean breaks per cell) in *185delAG* mutation carriers. For the CC and CT combined genotypes, the OR was also elevated (OR=8.0, 95% CI:0.6-106.9; p=0.14). There was not an allelic dose effect for the C allele and the highest OR was found for the CT heterozygote, so results could be due to population effect and could possibly be falsely positive. Additionally, in *5382insC* mutation carriers ORs for the variant alleles for *D693N* and *Q356R* were 0.4 (0.01-8.1) and 0.3 (0.01-6.40), respectively, showing that having these variant alleles was associated with low MBPC (mean breaks per cell), but results were not statistically significant (p=0.73 and 0.29, respectively). These results show that *BRCA1* polymorphisms could modify risk *BRCA1* mutation carriers.

When haplotypes were assessed for DNA repair capacity, analysis revealed that BRCA1 haplotypes were not associated with DNA repair capacity (p=0.17). Sample size and power could have limited this study's ability to see genotypic differences between high and low MBPC (mean breaks per cell) subjects; 1327 individuals with EBV-immortalized cell lines would be needed to have and OR of 1.5 with a power of 0.80 and 95% confidence. In previous publications, the *BRCA1 Q356R* and *D693N* polymorphisms and *BRCA1* haplotypes inconsistently showed a relationship with breast cancer risk (78, 80-83). Dunning et al were one of the first to identify *BRCA1* haplotypes and found differential frequencies between cases and controls, although not significantly different (78). When studied independently (not as haplotype), their data did observe different *Q356R* genotype distributions between cases and controls (p = 0.01), indicating that the R allele may be protective against breast cancer

(OR=0.75, 95% CI: 0.55-1.02). Durocher et al also found *P871L, K1183R*, and *S1613G* polymorphisms in complete linkage disequilibrium ($r = 1.0$) and found that the *P871L* showed differential frequencies in cases compared to controls ($p<0.002$) (79). However, subject mutation status could not be confirmed and therefore, it can be speculated that this finding could be due to the mixture of sporadic and hereditary breast cancer cases and population artifact. Cox et al also revealed an increased risk (OR=1.18, 95% CI: 1.02-1.37) associated with a specific *BRCA1* haplotype and showed a dose-dependent effect in homozygous carriers of this haplotype (OR 1.62, 95% CI 1.05–2.48) (81). However, tagging SNPs selected included intronic SNPs, which were not of interest to us. Freedman et al did not find evidence of significant associations between *BRCA1* haplotypes and cases, but no stratification for environmental interactions, such as hormonal factors, were done (82). Our findings are consistent other *BRCA1* haplotyping studies and show that there is no evidence *BRCA1* haplotypes are associated with risk due to deficient DNA repair capacity.

Rad51 and DNA repair

The MSA (mutagen sensitivity assay) data was also used to assess the relationship between mutagen sensitivity and DNA repair capacity in *Rad51*. For *Rad51,* only functional SNPs in the 5' untranslated and coding (exonic) regions were examined. *Rad51* contains only 8 coding exons and thus resequencing for all exons was feasible. Additionally, most exons contained functionally important regions. For example, exon 3 contains a nuclear localization signal important for BRCA1 binding and colocalization. Exon 4 binds ssDNA. Exon 7 contains the region where *Rad51* binds itself to form heptamers. Exon 1 contains the 5' untranslated

region. However in this study, this region was genotyped because a polymorphism associated with DNA repair efficiency was found in *BRCA1* and *BRCA2* mutation carriers, namely the 5'UTR 135G>C (rs1801320) and 173 G>T (rs1801321).

Rad51 was resequenced to determine if this population had SNPs not published previously and not revealed in the HapMap for a set of subjects from a different population. To date, neither the HapMap website nor the NCBI websites, lists polymorphisms with greater than 5% frequency and lists only 2 with less than 5% frequency in exons 4 and 9 (rs7174493 and 11544205). Sequencing results of *Rad51* were consistent with other published results of *Rad51* coding region sequencing. *Rad51* has been shown to be highly conserved with few, if any, polymorphisms. Few polymorphisms were found and confirmed and none had a frequency of >5%. So these polymorphisms could be mutations and warrant more investigations. The two SNPs identified above were not found and results could not be replicated in our population.

The *Rad51* 5'UTR 135G>C SNP was examined in our population of high-risk individuals from breast cancer families. Because there were few differences in relationships for those with CC and CG genotypes, these categories were collapsed and the associations were evaluated between genotype and DNA repair efficiency. Results showed that the *Rad51* 5'UTR 135G>C allele was found to be associated with the MSA (mutagen sensitivity assay). The *Rad51* 5'UTR C allele also showed higher risk for higher MBPC (mean breaks per cell) among both *185delAG* and *5382insC* mutation carriers (OR=3.70 96%CI: 0.40-36.60 and OR=2.0 95%CI:0.10-26.70, respectively), but again, results were not statistically significant (p=0.21 and 0.42, respectively). To date, the *Rad51 5'UTR 135 C* allele has had positive findings in mostly *BRCA2* mutation carriers (86-88, 192), but contrary to our findings, two studies did find a

decreased risk of breast cancer to be associated with the variant C allele in *BRCA1 5382insC* mutation carriers. Specifically, these studies revealed that women who carry the variant C allele have a reduction in breast cancer risk compared to those with the wild-type allele (OR=0.23 95% CI 0.07-0.62, p=0.0015 and OR=0.57, 95% CI: 0.33-0.99, p=0.046) (100, 103). This study only had sixteen 5382insC mutation carriers, whereas the other studies were matched case-control with 83 and 181 pairs of 5382insC mutation carriers. We can only speculate why this change would decrease DNA repair capacity, but it has been suggested that mRNA stability and/or translation efficiency, could be the reason for such a change. Because an association between DNA repair capacity and the *Rad51* variant C allele in the high risk *BRCA1+* subjects was found, we decided to test the hypothesis that the same C allele was associated with risk in sporadic breast cancer. Our results, however, did not indicate that the *Rad51* 5'UTR 135G>C SNP was associated with breast cancer risk in the population-based case-control study of sporadic breast cancer. Exposures and gene-environment interactions were not studied, and so the conclusions from this study can only be limited to risks of the SNP as a main effect. This SNP has been previously studied for sporadic breast cancer, and null results have been reported (101, 102). However, one of the studies lacked power (n=46 cases and 66 controls) and was considered preliminary analysis to justify a larger study (101), whereas the other study contained enough power to detect differences among cases and controls (n=1456 cases and 793 age-matched controls), but failed to detect differences between breast cancer cases and controls (102). Elevated and decreased ORs, although not statistically significant, warrant additional examinations in both hereditary and sporadic breast cancer studies. These results suggest that

the *Rad51* 5'UTR 135G>C SNP may be modifying genotypes for the penetrance of *BRCA1* mutations in high risk families.

Strengths and Limitations

There are several strengths of this study. First, this study capitalized on a unique resource of EBV-immortalized cell lines from a large number of *BRCA1* mutation carriers, where subjects with concordant fresh peripheral blood lymphocytes were used to validate the MSA (mutagen sensitivity assay). Most of these studies use PHA-stimulated lymphocyte cultures from whole blood. However, in this study, we showed that the use of the MSA (mutagen sensitivity assay) in EBV-immortalized cell lines is a valid method with good laboratory reproducibility, and provides similar effects as whole blood cultures. Additionally, these studies indicate that for this assay, EBV immortalization does not affect the cell sensitivity to gamma irradiation and that MBPC (mean breaks per cell) results in EBV-cell lines compared to fresh blood was not statistically different ($p=0.11$).

An additional strength to this project was in the methods that were used to develop *a priori* hypothesis for SNPs to be examined in population-based epidemiological studies. Here, DNA repair capacity was assessed through studies of functional assays in individuals from high-risk families. Whereas, most studies examine relationships between risk and genotype, instead, this project looked at a biomarker or phenotype (MBPC (mean breaks per cell)) for the exploration of mechanisms for decreased DNA repair and possibly increased breast cancer susceptibility. The use of a functional *in vitro* assay allowed the development of *a priori hypotheses* to identify genetic variants that may be associated with DNA repair capacity and

possibly risk. Thus, it was hypothesized that polymorphisms and haplotypes in double strand break repair genes could affect DNA repair capacity and increase susceptibility to breast cancer resulting from deficient DNA repair. While, the first part of the study lacked power to detect genotypic differences in some of the SNPs, positive results were still obtained which allowed for the selection of that SNP to be further assessed in a large, well-designed case-control study. These methods aided in the identification of SNPs possibly related to breast cancer risk and prevented "fishing" for discovery of SNPs that have no relevance for disease risk. Without *in vitro* functional studies, a connection between a genotype and risk can rarely be made. Associations that lack this component, the use of *a priori* hypotheses, rarely yield positive results and when they do, they are likely due to chance or results yield false-positives (17, 18).

The evaluation of multiple end points is another strength of this study. Both DNA repair capacity and risk was evaluated. First DNA repair was evaluated in high-risk individuals to develop an *a priori* hypothesis to then evaluate risk. A relatively large sample of BRCA1 mutation carriers were available, which aided in the development of *a priori* hypotheses. The combination of these methods and strategy, a strong model was developed for the assessment of SNPs in large breast cancer epidemiological studies.

The use of a well developed case-control study for the breast cancer risk assessment of SNPs was and additional strength to this study. Although, results were null in sporadic breast cancer, this is one of the first studies, to our knowledge, to show the relationship between *Rad51*, BRCA1, and DNA repair. The use of case-control studies for the assessment of cancer risk has proven globally valuable and has established evidence for prevention and treatment strategies.

Along with strengths, this study also has some limitations. Again, a small sample size was available for the MSA (mutagen sensitivity assay) subset analyses, where genotypes and haplotypes occur at low frequency. Thus, statistical power is limited and there may still be effects of the genotypes considered herein, not observable with small numbers. Sample size and power calculations were done and it was shown that in order to get higher than an OR of 2.0 with a power of 0.80, at least 419 subjects would be needed. Below, Table 2 shows the sample size and power needed for specific ORs.

Table 2. Sample size and power calculations for BRCA1 and *Rad51* SNPs

Polymorphism	α	*n*	Po (variant in lows/ # lows)	*m* (low/high)	Ψ/OR	β
E1038G	0.05	110	0.54	0.67	1.21	0.128
D693N	0.05	110	0.02	0.64	5.98	0.62
Q356R	0.05	110	0.26	0.66	0.54	0.39
Rad51 135g>c	0.05	110	0.21	0.66	3.4	0.96
Rad51 135g>c	0.05	203	0.05	1.5	3.4	0.96
Sample size calculations	***0.05***	***1387***	***0.05***	***1.5***	***1.5***	***0.8***
	0.05	***1870***	***0.05***	***1.5***	***1.5***	***0.9***
	0.05	***2322***	***0.05***	***1.5***	***1.5***	***0.95***
	0.05	***419***	***0.05***	***1.5***	***2***	***0.8***
	0.05	***219***	***0.05***	***1.5***	***2.5***	***0.8***
	0.05	***142***	***0.05***	***1.5***	***3***	***0.8***

Another limitation is that the volunteers enrolling in the FCR may not representative of women from high risk families in particular, or even in BRCA1 mutation families. This population may have a distribution of observed phenotypes different from that of the general population. However, frequency distributions were similar to those reported in the general population. And when specific mutations were compared in the MSA (mutagen sensitivity assay), results showed no correlations or significant differences between the 5382insC, 185delG, and other mutations and DNA repair capacity. The same was even shown with the C61G

mutation. This demonstrates that these mutation carriers may start off on "equal" footing compared to the general population when DNA repair is concerned. The concept of BRCA1 mutation carriers already being DNA repair compromised, although plausible, might have been presumptuous. This study was consistent with other showing that affected individuals have increased MBPC (mean breaks per cell), but was results were not affected by mutation status.

Also, in this study, subjects were volunteers to the registry without regard to treatment. Thus, some subjects were enrolled after chemotherapy or radiotherapy. While this might alter lymphocyte response and the MSA (mutagen sensitivity assay), there was no indication that this occurred, and the results were comparable to prior reports in the literature compared women with and without breast cancer (see Table 1, Chapter 2).

Another limitation was that the Lombardi Comprehensive Cancer Center Familial Cancer Registry is a voluntary registry of referred women from high risk families and subject accrual was not done systematically; it is unknown how these registry volunteers compared to other women from breast cancer families; we were not able to examine comparisons for women who did and did provide blood to the FCR. Also, although we had access to records and epidemiological questionnaire data, many were incomplete and/or different. The use of three different questionnaires was found in the familial study of hereditary breast cancer so we could not do assessment of non-genetic factors, such as lifestyle and exposure factors that could've been determined to study gene-environment interaction. Also, while many records indicated that affected cases with breast cancer had been treated with chemotherapy or radiotherapy, the dates for these treatments were not provided. Moreover, the use of HRRT and chemo- or radiation

therapy could just be answered with a simple yes or no. For these types of studies, it is very important to know length of use and dosage. Because this was one of the first studies where EBV-immortalized cell lines from BRCA1 mutation carriers from the Lombardi Comprehensive Cancer Center Familial Cancer Registry were used, these issues could have not been predicted and these types of considerations were not initially made. However, these issues are being actively addressed and more comprehensive epidemiological questionnaires have been developed. Additionally, medical records are being accessed to complete questionnaires and data from all of the questionnaires are being merged together where a more complete database should be available in the future for the assessment of risk in these subjects.

Of subjects were currently being treated or recently treated, it is conceivable that the MSA (mutagen sensitivity assay) might be affected due to changes in blood lymphocytes. For the MSA (mutagen sensitivity assay), MBPC (mean breaks per cell) is determined by the count of chromosomal breaks in 50-100 metaphases. For some subjects, less than the required amount of metaphases could be obtained. Of the 138 subjects, 43 (31%) did not have 50 metaphases. Specifically 41 (73%) of the unaffecteds and 54 (66%) of the affecteds had 50 metaphases. However, subjects with less than 50 metaphases were not more likely to be affected ($\chi2=0.22$, $p=0.64$) or have high MBPC (mean breaks per cell) ($\chi2=0.71$, $p=0.39$). Additionally, dichotomization of values into high and low MBPC (mean breaks per cell) helps to deal with extremes and outliers, if any. It is conceivable that these subjects were recently treated prior to blood collection.

Initially pair-wise tagging methods were used to identify tag SNPs. This method involves identifying the least amount of SNPs possible to get maximum information about other genotypes. For example, in BRCA1, one SNP can be used to identify 20+ genotypes. Pair-wise tagging methods are very tedious and should only be used for small populations that may differ from the general population.

HapMap and haploview were also used for the identification and selection of tag SNPs. Haploview LD plots showed that haplotypes from the studied population deviated slightly from the general Caucasian population. For example, in the general Caucasian population, the *D693N* SNP appears to be in high linkage disequilibrium (LD) with other SNPs *E1038G* and *K1183R*. On the other hand, our population's LD plot clearly demonstrates low LD between these same SNPs. However, the haplotype tagger in Haploview, for Caucasians, identified the same 3 tag SNPs in our population. The *E1038G*, *Q356R*, and *D693N* SNPs were used for genotyping and haplotyping analysis.

Overall Conclusions

In summary, the aim of the present study was to attempt to elucidate a role for genetic variants as modifiers for breast cancer predisposition in *BRCA1* familial breast cancer patients and breast cancer risk in the general population. After validating the MSA (mutagen sensitivity assay), there was a wide variability for MBPC (mean breaks per cell) in these study subjects. Additionally, there was an increased number of MBPC (mean breaks per cell) in BRCA1 carriers with breast cancer compared to those without breast cancer. In this study, we found a significant association between the *Rad51* 5'UTR 135G>C polymorphism and DNA repair capacity, but did not find any significant associations between BRCA1 SNPs or haplotypes and DNA repair

capacity. For sporadic breast cancer, the *Rad51* 5'UTR 135G>C polymorphism did not predict risk. This study provides data to justify a well-designed epidemiology study of the MSA (mutagen sensitivity assay) as a predictive phenotype in *BRCA1* mutation carriers and breast cancer risk. The results indicated that the *Rad51 5'UTR 135C* allele and maybe the *D693N* allele are modifying genotypes for the penetrance of *BRCA1* mutation carriers and so might only be risk factor for high risk families.

This study contributes to other existing data on the use of EBV-immortalized cell lines for DNA repair assays such as the Mutagen Sensitivity Assay. It also provides more evidence for the function of *Rad51* and BRCA1 in DNA repair with the use of an *in vivo* functional DNA repair assay.

Future plans

In the future, other genes in DNA repair pathways should be tested to see if they are associated with 1) DNA repair in this population in high-risk individuals and then 2) in larger case-control studies. Also, because we found an elevated risk for decreased DNA repair capacity for the *D693N* genotype, it would be interesting to see if it is ultimately associated with risk. Very few studies have examined the relationship between the BRCA1 *D693N* variant genotype and even haplotype analysis of BRCA1 has failed to identify this SNP as a tagging SNP (82).

The MSA (mutagen sensitivity assay) information established here could also help in the development of *a priori* hypotheses for the studies of other genes involved in DNA repair and could help identify possible variants that could plausibly affect breast cancer risk. Genes that would benefit from this could be other genes involved in HRR, such as *Chk1, Chk2, ATM, ATR, BRCA2, XRCC2, XRCC3, Rad52, Rad53*, and etc. Although variants in the genes listed above

were not found to be associated with risk in the general population in a genome-wide scan and association study, variants in these genes could affect risk in individuals from high-risk families or individuals with *BRCA1* and *BRCA2* mutations. Ultimately, gene-gene interaction analyses could discover why some women in this group get breast cancer and others don't. (141-143, 177, 179, 180, 182-186, 193-201)

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Materials and Methods

Subjects from the Familial Cancer Registry.

This study received approval by the institutional review board at Georgetown University and participants were recruited as a part of the Lombardi Cancer Center Familial Cancer Registry Study. A detailed description of ascertainment, eligibility, and participation rates have previously been published (148). Subject demographics can be seen in the results section. In short, following informed consent, subjects provided blood samples and answered interview questions that could be used for future studies of genetics and cancer risk. All subjects are members of high risk breast cancer families, with a family history of breast cancer, and have had genetic analysis for BRCA1 and BRCA2 carrier status. Specifically, subjects were recruited under two separate protocols: CARE and SAGE. CARE is the genetic counseling protocol through which the vast majority of registry participants were recruited (n=124). Generally, the first person tested in a family through CARE must have been diagnosed with either breast or ovarian cancer. Family members are then recruited if there is a family history of breast or ovarian cancer. SAGE is the genetic counseling protocol for women newly diagnosed with breast cancer (n=14). Women in SAGE undergo BRCA1/2 testing before definitive surgery. For all subjects, whole blood was cultured and the lymphocytes were immortalized (details below), and cryo-preserved. For this study, a subset, "high-risk" individuals, were collected (n=149), based on the proband case being a BRCA1 mutation carrier. Of the subjects who met study eligibility (Caucasian females with enough metaphases) and were analyzed (n=138), 82 are affected with a history of cancer and 56 are unaffected (never had cancer) *BRCA1* mutation

carriers but from the families with a mutation carrier. Of those 138 cell lines obtained, 5 were BRCA1/2 negative, 7 were Jewish panel negative, 9 were true negatives, and 117 were BRCA1 mutation carriers (73 affected and 44 unaffected). For Ashkenazi Jewish subjects, genotyping for founder mutations were done at the Clinical Molecular Diagnostics Laboratory at Georgetown University. BRCA1/2 negative, Jewish panel negative, and true negatives were included in the final analysis because all were members of "high-risk" families. BRCA1/2 negative individuals underwent full sequencing for BRCA1 and BRCA2 mutations, but no deleterious mutation was identified. This individual may carry a mutation in BRCA1 or BRCA2 that is not detectable by current testing, or this individual may carry a deleterious mutation in an unidentified cancer susceptibility gene. Jewish panel negative subjects tested negative for the 3 founder mutations found in individuals of Ashkenazi Jewish descent (185delAG, and 5382insC). True negatives were tested for a mutation already identified in her family, and she was found not to carry that mutation. In this population, 48 (34.78%) subjects were related.

Phlebotomy was performed by trained personnel at the Georgetown University Medical Center. One green-top tube (sodium heparin solution) was obtained for fresh culture and the mutagen sensitivity assay (MSA), and one yellow-top tube (ACD) was obtained for EBV-immortalization. Twenty additional fresh blood samples were received, labeled, and logged into a secure database.

Peripheral Blood Lymphocyte Collection for MSA (mutagen sensitivity assay) Validation

Phlebotomy was performed by the Georgetown University Medical Center General Clinical Research Center for subjects enrolled in the Familial Cancer Registry. For subjects

participating in the MSA (mutagen sensitivity assay) validation Aim, two green-top tubes were obtained, one of which was used for fresh culture and the mutagen sensitivity assay (MSA), and the other for EBV-immortalization of lymphocytes. Other participants had been phlebotomized according to the Registry usual protocols that included one green-top tube for EBV-immortalization. Fresh blood (3-10 ml) from 19 female BRCA1 mutation carriers for Aim 1 was immediately delivered by a phlebotomist. Two milliliters of blood was used for the mutagen sensitivity assay and rest of the blood was spun down in a CPT tube for a minimum of 25 minutes at 2500G to separate lymphocytes from red blood cells. The lymphocytes were then aliquoted with cell freezing media (GIBCO #11101-01) in a cryo-centrifuge tube and stored in the gaseous phase of liquid nitrogen. Whole blood cell cultures, as described below, were first cultured with 24 hours of phlebotomy.

Cell Lines

Cryopreserved EBV-immortalized lymphocyte cell lines were obtained from the Familial Cancer Shared Resource at the Lombardi Comprehensive Cancer Center. A total 149 EBV-immortalized cell lines were obtained from Georgetown University's Tissue Culture Core, but only 138 had readable metaphases, and met eligibility inclusion criteria. The other 11 were males or females of other races. The Tissue Culture Shared Resource (TCSR) assists Lombardi Cancer Center investigators and other members of the Georgetown University Medical Center research community with the tissue culture related aspects of their research. The TCSR manages Lombardi's cell line repository, which includes an extensive collection of normal and tumor cell lines (animal and human) stored in a series of liquid nitrogen freezers. These cell lines are made

available to investigators either as frozen vials of cells or as growing cultures so our cell samples were either delivered in a flask containing live cells in 10 mL of media or as a frozen cell pellet containing around 1 x 10^6 cells.

The EBV-immortalized lines were established by the TCSR using previously described methods (149-151). Briefly, equal amounts of blood and PBS (Mediatech, Inc, VA) were slowly added to a tube filled with ficol (Amersham Biosciences, Sweden) to obtain clear separation of blood components. The mixture is centrifuged and the plasma layer was discarded, whereas the lymphocyte layer is transferred to a tube containing EBV virus supernatant (ATCC, VA), Cyclosporin A (Biomol International LP, PA) and RPMI1640 medium, supplemented with 10% fetal calf serum (Sigma, MO), 2% L-glutamine (GIBCO, CA), 1% Sodium Pyruvate (GIBCO, CA), 1% NEAA (non-essential amino acids-GIBCO, CA), 0.1% 2-mercaptoethanol (GIBCO, CA), and 0.1% gentamycin (Invitrogen, CA). After several media changes, the tubes are then incubated at 37°C in 5% CO_2. The cell culture media was changed once a week when cell pellets were visible or when cell growth was of sufficient quantity to cause the media to become acidic after 72 hours. Cell pellets were kept in cell culture freezing media (GIBCO #11101-011) and stored in liquid nitrogen by Georgetown University's Tissue Culture Core.

Subjects for Case-Control Study

Dr. Jo Freudenheim at the State University of New York at Buffalo, our collaborator, has enrolled 1165 breast cancer cases and 2170 controls in a population-based case-control study of breast cancer and diet. Individuals included in this analysis were participants in the Western New York Exposures and Breast Cancer (WEB) study, a large, population-based case-control

study conducted between 1996 and 2001. This study has been described in detail elsewhere and subject characterization can be seen in the results section. (152). Briefly, subjects are all residents of Erie and Niagara counties, New York. Cases are women with incident breast cancer between the ages of 35-79 years. Controls were randomly selected from residents of Erie and Niagara Counties using lists provided by the New York State Department of Motor Vehicles driver's License enrollees for those less than 65 years of age, and the Health care Finance Administration for those 65 years of age and older. Controls were frequency matched by age, sex, race, and county of residences to cases. The protocol was approved by the University at Buffalo Institutional Review Board, as well as by the review boards of the participating hospitals. Informed consent was obtained from all study participants. A detailed interviewer-administered questionnaire was used to assess use of postmenopausal hormone replacement therapy and other breast cancer risk factors. Blood and oral rinses from cases and controls have been obtained and DNA was extracted by phenol-chloroform methods. DNA aliquots were made, labeled, logged in a secure database, and store in -20°C.

Mutagen Sensitivity

The mutagen sensitivity assay was performed on 138 EBV-immortalized cell lines and peripheral blood samples. Lymphocyte cultures were established by mixing fresh whole blood (1 ml) with 9ml RPMI1640 medium (GIBCO, CA) supplemented with 20% fetal calf serum (Sigma, MO), and phytohemagglutinin (112.5 μg/mL; GIBCO, CA), were incubated at 37°C in 5% CO_2. The EBV-immortalized lymphoblastoid cells were cultured similarly, except it was supplemented with 10% fetal calf serum (Sigma, MO), 2% L-glutamine (GIBCO, CA), 1%

Sodium Pyruvate (GIBCO, CA), 1% NEAA (non-essential amino acids-GIBCO, CA), 0.1% 2-mercaptoethanol (GIBCO, CA), and 0.1% gentamycin (Invitrogen, CA). Fresh media was made once a week and at the time of the assay, EBV-immortalized cells had undergone less than 15 population doublings.

After 67 hours of culture, the MSA (mutagen sensitivity assay) was performed by irradiating the cells with 1 Gy gamma radiation (^{137}Cs source gamma irradiator), according to the method of Sanford and Parshad (153, 154). After incubating an additional 4 hours, the cultures were treated with 0.04ug/ml colcemid (GIBCO, CA) to arrest the cell cycle. The cells were then treated with hypotonic solution (0.06 mol/L KCl; Sigma, MO) and then fixed [3:1 methanol (Sigma, MO):glacial acetic acid (Fisher, PA)] and metaphase spreads were established by dropping 2-3 drops of fixed cells on clean slides, then allowed to air dry for up to 48 hours. Next, slides were stained with Giemsa (Sigma-Aldrich Corp., MO) for 10 minutes and cells arrested in metaphase with good spreads were evaluated under light microscopy. The frequency of chromatid breaks per cell (b/c) was calculated as a measure of an individual's DNA repair efficiency. The definition of a chromatid break is discontinuity longer that the chromatid width. A minimum of 25 and a maximum of 50 well-spread, clear, and complete metaphases per culture were scored. Readings were performed blinded to subject, replicate and paired-sample status.

DNA Isolation

Genomic DNA was extracted from the EBV-immortalized cell culture pellets in the gaseous phase of liquid nitrogen using 2 different methods. In the first method, the Qiagen M48 Biorobot (Qiagen, # 9000708) was used to isolate DNA from cultured cells using the

MagAttract® DNA Mini M48 Kit (Qiagen, #953336). In short, <2 x 10^6 fresh or frozen cells were used. First, the cell pellet was incubated at 56°C overnight in 190uL of cell lysis solution with 10 μl of Proteinase K to digest to proteins in the cell pellet. Then the tube was placed in the M48 biorobot, where the reagents unidentified reagents (i.e. Buffer 1, Buffer 2, etc.) were placed in containers. Briefly, the QIAsoft M operating system was launched, and the "Cultured Cells" protocol was selected. Elution tube type, the number of samples, and sample and elution volumes were selected. All the steps were fully automated, and the software message on the screen indicated when the procedure was finished. Once, the procedure was finished, the elution tubes containing the purified DNA were retrieved from the cooling block. Because the DNA was to be analyzed by PCR, a magnetic separator was applied to the eluate to minimize the risk of magnetic-particle carryover. The clean eluate was transferred to a clean labeled 2.0uL tube.

DNA extraction also was performed using Phenol-chloroform-isoamyl alcohol methods as previously described (155). Briefly, 20mg/ml Proteinase K was added to each sample and samples were place in a heat block until all cells were digested. To inactivate Proteinase K, samples were then heated to 95°C for 10 minutes. Then, 35 μl of RNAse A (10mg/ml; Invitrogen, CA) was added to samples and were then incubated at 37 C for 1.5 hours. Three hundred microliters of phenol:chloroform:isoamyl alcohol (25:24:1; Sigma, MO) was added to samples, then vortexed for 15 seconds, followed by 5 minutes of centrifugation at 14000rpm. The aqueous layer was transferred to a new tube and the above procedure (addition of phenol:chloroform) was repeated again. The aqueous layer was transferred to a new tube and 50uL of 10M ammonium was added along with 6 μl of glycogen (5mg/ml; Invitrogen, CA) and

600 µl of 100% ethanol was added before samples were placed at -20°C overnight. After removing from -20°C, samples were centrifuged for 15 minutes at 14000 rpm. The supernatant was carefully removed without disturbing the DNA pellet. The pellet was then washed with 70% ethanol and centrifuged again for 5 minutes at 14000 rpm. The sample was then dried and resuspended in 50 µl of sterilized, filtered, DNAse-free water (Biosource, Rockville, MD) and stored at 4°C.

DNA Quantification

DNA concentration were determined and quantified by the SPECTRAmax Plus Spectrophotometer (Molecular Devices, Union City, CA). In brief, 98 µl dH_20 was added to 2 µl DNA in a 96-well Costar UV plate, with several wells acting as blanks. The plate is then inserted into the machine, where the average OD calculated with absorptions at 260nm/280nm, and DNA concentration and quality is determined.

Rad 51 Sequencing

Rad51's coding region was sequenced using pre-designed ABI Variant SEQr primers (Applied Biosystems, Foster City, CA; RSS ID 000015033_02) on subjects who had the highest and lowest mean breaks per cells (n=92). Exon primer sequences are listed in Table 1.

Table 1. Primer and Probe sequences for genotyping and sequencing.

Exon	RSA ID	Forward	Reverse
Exon 2	RSA000470002	TGTAAAACGACGGCCAGTGGCACAATAAGAGAATGGCCTTGG	CAGGAAACAGCTATGACCGGAAGAGGCCCTGCCAGACA
Exon 3	RSA000737142	TGTAAAACGACGGCCAGTGGCTCTCTGTATCTCCGGATTCTG	CAGGAAACAGCTATGACCTCCCACTAATGCCTCCCTAGGTT
Exon 4	RSA000017906	TGTAAAACGACGGCCAGTCCCAAGGATTTCAAGGGACAGTTG	CAGGAAACAGCTATGACCCCTGGAAAGGATAATGAGATGACCG
Exon 5	RSA000017907	TGTAAAACGACGGCCAGTTTCTCTTCCCATTGCACACCTTG	CAGGAAACAGCTATGACCTGGAAGCTTTCCTAACTAGAGTTCACA
Exon 6	RSA000068339	TGTAAAACGACGGCCAGTCCACCACGGCTAGCCTCAAA	CAGGAAACAGCTATGACCTCTGATAGGAGATCAATGATTTGGAAG
Exon 7	RSA000014020	TGTAAAACGACGGCCAGTGGGAATGCCTCCTTCCTACCA	CAGGAAACAGCTATGACCTTGAACCACACATACAACTTTGGGA
Exon 8	RSA000579764	TGTAAAACGACGGCCAGTGAAAGGGAAATACATTCTTTGGTCCTG	CAGGAAACAGCTATGACCCCTTGTGATCCACCCGCCTT
Exon 9	RSA000068341	TGTAAAACGACGGCCAGTACTCCGGAGGCTGAAGCAGG	CAGGAAACAGCTATGACCAATGCTGCCACCACTTTCCC
Exon 9	RSA000571422	TGTAAAACGACGGCCAGTGGCTGTGAATTCCAGGAGCCTT	CAGGAAACAGCTATGACCCACTGTGCCCAGCCCAAACA
Exon 10	RSA000788802	TGTAAAACGACGGCCAGTTCAATATGGACAATCCACTGAGAGGG	CAGGAAACAGCTATGACCTCCCGGAAGCTTTATCCTGGC
Exon 10	RSA000014026	TGTAAAACGACGGCCAGTGCAGATGGAGTGGGAGATGCC	CAGGAAACAGCTATGACCTGGTGAAGGGCTAATGGCCT

PCR reaction

To begin sequencing of *Rad51*, PCR reactions were prepared. In short, a master mix composed of AmpliTaq Gold® PCR Master mix (2X; Applied Biosystems #58004012-01, Foster City, CA), sterile, deionized water, 50% glycerol (Sigma #G8778) and VariantSEQr ™ RSA primer mix (Applied Biosystems, Foster City, CA) were combined. This master mix (9ul) was added to designated wells in a 96-well plate. Five to ten nanograms of DNA was then added; the plate was sealed, vortexed briefly, and centrifuged at 1600g for 30 seconds. The reaction was run on the GeneAmp® PCR system 9700 and the thermal cycler program was as follows: activation at 96°C for 5 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 45 seconds and 72°C for 45 seconds; Extension at 72°C for 10 minutes.

Removal of unincorporated dyes and nucleotides

Five microliters of PCR product was treated with 1uL of ExoSAP-IT (Exonuclease I and Shrimp Alkaline Phosphatase- USB 7820) and un-incorporated PCR primers and nucleotides were degraded and inactivated via treatment. The mixture was then vortexed briefly and incubated at 37°C for 15 minutes. Then the ExoSAP-IT was inactivated by heating at 80°C for 15 minutes. Finally, the PCR product was ready for direct sequencing. PCR products treated with ExoSAP-IT were stored at -20°C until used. The yield for a robust PCR was around 25-50ng/ul. Quantity estimate of PCR product was done by running on 2% agarose gel accompanied by Hyperladder II (Bioline BIO-33039).

Sequencing Reaction

For the sequencing reaction, 10-20ug of PCR product was used (approximately 1-2ul final PCR volume). The amount of template used was very important. Experience showed that the use of the minimum possible amount of template was best. To prepare the sequencing reaction, it was important to use the recommended quantities of double-stranded PCR product template: < 1000bp – start with 5 - 50ng, and >1000bp – start with 10 - 100ng. The sequencing primers were diluted to 5 μM (5 pmole/μl) concentration with water. When the PCR product was ready for direct sequencing, 5 pmole/μl M13 Universal Forward Primer (5'TGTAAAACGACGGCCAGT-3') and DYEnamic ET terminator reagent premix (Amersham Biosciences #US81090) were added to the clean PCR product. When all reagents had been dispensed, the cocktail was mixed thoroughly by gentle pipetting. The reaction plate was centrifuged briefly to bring contents to the bottom of the tube or well. The plates were sealed and placed into the pre-programmed thermocycler (30 cycles of 95°C, 20 s; 50°C, 15 s; 60°C, 1

min). The total volume of the reaction mix was 20.0 µl. After cycling was complete, the plates were centrifuged briefly to collect the reaction mixtures at the bottom of the tubes/wells.

Post-reaction clean up and sequencing

For the post reaction clean-up, an AutoSeq96 plate (Amersham Biosciences #27-5340-10) was used to get rid of un-incorporated dye. The plates were stored at 4ºC (for up to 8 months) and allowed to come to room temperature (at least 2 hours) before use. One hundred fifty microliters of dH_20 was added to each well of AutoSeq96 Plate and assembled top of NUNC Nunclon Surface 96-well collection plate (NUNC #163320). The assembled unit was centrifuged with swinging 96-well plate carrier for 5 minutes at 910 x g. This step was done twice; then the NUNC collection plate containing the dH_20 was removed and replaced with an Abgene AB-0800 96-well plate. Slowly, 20.0µl of the sequencing reaction was applied to the center of each column resin bed in the wells of the AutoSeq96 plate and the assembled unit centrifuged for 5 minutes at 910 x g. The sample plate was then loaded in the MegaBACE 1000 for capillary sequencing (MegaBACE 1000, GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Sequence analysis

Sequencher software (Sequencher 4.7, Gene Codes Corporation, Ann Arbor, MI) was used to determine the DNA base sequences and variants. Sequences were lined up against wild-type sequences provided by the NCBI website (http://www.ncbi.nlm.nih.gov) and when variation was encountered, chromatogram was examined thoroughly.

Quality Control

More than 20% percent of the sequences were repeated for quality control. Mutations were confirmed by running remaining PCR product in the reverse direction with M13 Universal reverse primer (5'CAGGAAACAGCTATGACC-3').

BRCA1 and Rad51 Genotyping

Rad51 and *BRCA1* genotyping was done by amplification with PCR-based assays. Genotyping was carried out using the ABI/Biosystems Taqman methods (Applied Biosystems, Foster City, CA) as previously described (86), and primers are listed in **Table 2.** Briefly, TaqMan® Universal PCR Master Mix (Applied Biosystems #4304437; Foster City, CA) and TaqMan ® SNP Genotyping Assay Mix was combined and added to a 384-well plate containing 5-10ng of Genomic DNA in each well for a total volume of 5.0 µl. The reaction plate was centrifuged briefly to bring contents to the bottom of the tube or well. The plates were then sealed and placed into the ABI Prism 7900HT (50°C, 2 minutes; 95°C, 10 min and 49 cycles of 92ºC, 15 s and 60°C, 1 min). The basis for PCR quantitation in the ABI Prism 7900HT instrument is to continuously measure PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe called a TaqMan® probe. This probe is composed of a short (ca. 20-25 bases) oligonucleotide that is labeled with two different fluorescent dyes. On the 5' terminus is a reporter dye and on the 3' terminus is a quenching dye. This oligonucleotide probe sequence is homologous to an internal target sequence present in the PCR amplicon. When the probe is intact, energy transfer occurs between the two dyes and emission from the reporter is quenched by the quencher. During the extension phase of PCR, the probe is cleaved by 5'

nuclease activity of Taq polymerase thereby releasing the reporter from the oligonucleotide-quencher and producing an increase in reporter emission intensity. Results were also analyzed using the ABI Prism 7900HT where SDS 2.1 (Foster City, CA). The Sequence Detection Software v2.1 (SDS 2.1) examines the fluorescence intensity of reporter and quencher dyes and calculates the increase in normalized reporter emission intensity over the course of the amplification. The results are then plotted versus time, represented by cycle number, to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification plot reaches the threshold (defined as the threshold cycle or Ct). Differences in threshold cycle are used to quantify the relative amount of PCR target contained within each well.

Table 2. Primer and Probe sequences for *Rad51* and *BRCA1* polymorphisms.

Gene	SNP	rs#	Forward	Reverse	Probes
Rad51	5'UTR 135G>C	1801320	GCAGCCTCCTCTCTCCAGC	GCTGGGAACTGCAACTCATCT	FAM-CCCCAACGCCCCTGGCTTAC
					VIC-CAACGCCCGTGGCTTACGCT
BRCA1	Q356R	1799950	ATCCCCTGTGTGAGAGAAAAGAAT	AAGGAACATCTTCAGTATCTCTAGGATTC	FAM-AGTTTCCGCTTATTC
					VIC-AGTTTCTGCTTATTCC
BRCA1	D693N	4986850	CCAAGAAGAGTAACAAGCCAAATGA	ACACTTAGTAAAAGAACCAGGTGCATT	FAM-ACAAGTAAAAGACATGACAG
					VIC-ACAAGTAAAAGACATAACAG
BRCA1	K1183R	16942	TGACATTAAGGAAAGTTCTGCTGTTTT	TGTATGGGTGAAAGGGCTAGGA	FAM-CCAGAGAGGAGAGCT

Haplotyping and Phasing

Haploview © (Haploview 3.32, Broad Institute of MIT and Harvard, Boston, MA) was used to pick *BRCA1* and *Rad51* tag SNPs. Haploview is an available program provided on-line through the International HapMap project (http://www.hapmap.org/). The International HapMap Project is a multi-country effort to identify and catalog genetic similarities and differences in

human beings. Using the information in the HapMap, researchers will be able to find genes that affect health, disease, and individual responses to medications and environmental factors.

Specifically, input files of BRCA1 and *Rad51* SNPs found in Caucasians were found in the Haploview website. Then, reconstruction of estimated haplotypes using population genotypic data was performed using PHASE (PHASE 2.1, Department of Statistics, University of Washington, Seattle, WA) software (156, 157). To run the program, an input file containing genotype information, where each genotype was characterized by a number (1=A, 2=C, 3=G, and 4=T) was written. The program outputs a summary of results, including haplotypes frequencies, list of most likely pairs, estimates of recombination parameters, and goodness of fit of estimated haplotypes to the underlying model.

Statistical Analysis

Peripheral Blood Lymphocyte and EBV-immortalized cell line mutagen sensitivity comparison

The mean number of chromatic breaks per cell (MBPC (mean breaks per cell)) was obtained by counting 25-50 metaphases. To compare mutagen sensitivity responses in PBL and EBV cell lines, spearman correlation statistics (ρ) were calculated for the mean breaks per cell from the PBL and EBV-transformed cell lines with SPSS 12.0 for windows (SPSS.com). To test the hypothesis that there is no variation between readings, the log transformation was utilized to normalize the data and the standard paired t-test was utilized using SAS 9.1 (SAS Institute Inc. 2004, Cary, NC). Furthermore, to compare the variation (dispersion of the standard deviation) and variability of the mutagen sensitivity responses within each subject (intra-individual variation) and between subjects (inter-individual variation), coefficients of variations were

calculated. An analysis of variance (ANOVA) was also used to compare the within- and between-subject variations. The independent variable was each subject and the dependent variable was mean breaks per cell and each day or reading.

Genotype-Phenotype Statistics

The association between genotype and the mean number of breaks has been investigated via logistic regression, and odds ratios of relative risk with 95% confidence intervals have been calculated. Two-sided *P*-values of ≤ 0.05 were considered as statistically significant. Next, these odds ratios were adjusted for age as it is a possible confounder. Mean breaks per cell were dichotomized as high or low with a threshold of 0.20, determined by the median average number of breaks per cell in unaffected subjects. On these dichotomized observations, the chi-square test was used to look at their relationship to SNPs. Mean breaks per cell were dichotomized as high or low with a threshold of 0.20, determined by the median average number of breaks per cell in unaffected subjects. On these dichotomized observations, the chi-square test was used to look at their relationship to SNPs.

Genotype frequencies in affected vs. unaffected subjects were compared using the chi-square test and a test of Hardy-Weinberg equilibrium was performed. Genotype frequencies were compared across groups using the chi-square test. Fisher's exact tests were used for 2×2 tables when a cell frequency was <5. To account for potential bias due to the presence of relatives, the analysis on all 138 subjects was supplemented with an analysis on 110 subjects where relatives were excluded. The association of disease status and polymorphisms was analyzed by unconditional logistic regression. Odds ratio estimates of relative risk with 95% confidence intervals were computed and adjusted for age.

For *Rad51* 5'UTR 135g>c genotyping, GC and CC genotypes were combined for regression analysis due to the low proportion of subjects with the CC genotype (<1%). Gene-gene interactions were also evaluated by means of logistic regression analysis. The statistical analyses were done using SAS/STAT ® software, version 9.1 (SAS Institute Inc., Cary, NC).

Genotyping Analysis in Case-Control Study

In the case-control study, allele frequencies in cases and controls were tested for Hardy-Weinberg equilibrium. Odds ratios of relative risk with 95% confidence intervals were calculated and adjusted for age education, BMI, age at first birth, age at menarche, age at menopause (for post-menopausal women only), number of births, first-degree relative with breast cancer, and previous benign breast disease. Two-sided *P*-values of ≤ 0.05 were considered as statistically significant.

Haplotyping Analysis

The association between haplotype and the mean number of breaks has been investigated via logistic regression, and odds ratios of relative risk with 95% confidence intervals have been calculated. Two-sided *P*-values of ≤ 0.05 were considered as statistically significant. Next, these odds ratios were adjusted for age as it is a possible confounder. Mean breaks per cell were dichotomized as high or low with a threshold of 0.20, determined by the median average number of breaks per cell in unaffected subjects. On these dichotomized observations, the chi-square test was used to look at their relationship to SNPs. Mean breaks per cell were dichotomized as high or low with a threshold of 0.22 MBPC (mean breaks per cell), determined by the median average

number of breaks per cell in unaffected subjects. On these dichotomized observations, the chi-square test was used to look at their relationship to haplotypes.

Power and Sample Size calculations

Power and sample size calculations were done using the PS Power and Sample Size Calculations program © (Version 2.1.30, Feb 2003) and the study design chosen to calculate power and sample size was a dichotomous independently-matched retrospective case-control, where the alternative hypothesis was expressed as an odds ratio. To calculate power (β), α was set at 0.05 and sample size at 110. P_0 is defined as the variant genotype frequency in subjects with low MBPC (mean breaks per cell), m was the ratio of low and high MBPC (mean breaks per cell), and Ψ (psi) was the OR found in our analysis. The sample size was also calculated with an Ψ (Odds Ratio) of 1.5, α of 0.05, P_0 (frequency of variant in controls) of 0.05, in a case-control study where there were 2 controls for every case (m=1.5), with a power (β) of 0.80, 0.90, and 0.95. When an association between genotype and phenotype was found (as in the case for *Rad51*), it was checked and verified in a larger case-control breast cancer risk study (n=3123). The association between genotype and cancer status (case or control) has been investigated via logistic regression, and odds ratios of relative risk with 95% confidence intervals have been calculated. Two-sided *P*-values of $\leq$0.05 were considered as statistically significant. Next, these odds ratios were adjusted for confounders such as age, education, age at menarche, age at first birth, parity, body mass index (BMI), history of benign breast disease, family history of breast cancer, cigarette smoking, and race.

AACR 2006 Abstract

Induced chromosomal aberrations, genetics, and pathology in hereditary breast cancer

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Genetic breast cancer risk factors for both sporadic and familial breast cancer may be elucidated through studies of women with *BRCA1* mutations or in women in high risk breast/ovarian cancer families. There is variable penetrance for breast cancer among women in families with known BRCA1 mutations, and we hypothesize that this might be due to genetic variants in the wild-type allele of BRCA1 or other DNA repair genes. These genetic variants might also affect sporadic breast cancer risk. To identify genotype-phenotype relationships in DNA repair-related genes using EBV-immortalized cell strains from women in the Lombardi Cancer Center Familial Registry by assessing radiation-induced DNA repair capacity. Seventy-five affected and 57 unaffected *BRCA1* mutation carriers with immortalized cell lines were analyzed in this study. Their mean ages are 44 and 46, respectively ($p<0.01$). Gamma radiation-induced chromosomal breaks were measured as a marker of DNA repair capacity. The method was validated by assessing intra-individual variation in EBV-immortalized and peripheral blood lymphocyte test. Concordant samples were analyzed and spearman correlation was determined. *BRCA1* and *BRCA2* gene sequences were available and reviewed for the presence of genetic polymorphisms in relation to the number of chromosomal breaks using the chi squared test and unconditional logistic regression. To confirm that EBV-immortalized lymphocytes had little intra-individual variation, separate cultures were established and repeated measurements showed a high correlation ($r^2=0.994$), and a coefficient of variation of 12.37%. We assessed the relationship for the assay in freshly cultured lymphocytes and EBV-immortalized cell lines and found a high correlation ($r^2=0.865$, $p=0.01$). There was wide inter-individual variation for mean breaks per cell among the 130 women. Affected women had a greater number of mean breaks per cell than unaffected women (OR=3.2; CI: 1.5, 6.7). An association was found between a rare *BRCA1* muatation (C61G) and DNA repair efficiency; five out of six subjects carrying this mutation had high mean breaks per cell. There was no association *BRCA1* polymorphisms (E1038, K1183, L771L, P871L, S1613G, S694S) or haplotypes and DNA repair efficiency (n=32). There was also no association between *BRCA2* polymorphisms (H372N, K1132K, N289H) and DNA repair efficiency (n=32). Subjects with high mean breaks per cell were 2-fold (OR=1.8; CI: 0.9-3.6; $p=0.043$) more likely to be diagnosed with infiltrating ductal carcinoma and 4 times (OR=3.9; CI: 1.3-11.6; $p<0.01$) more likely to be diagnosed with metastatic breast cancer compared to subjects with low mean breaks per cell. The variable penetrance for breast cancer risk among BRCA1 carriers were not explained by common polymorphisms in BRCA1 or BRCA2, or haplotypes of BRCA1 in this model.

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LUISEL J. RICKS-SANTI

OBJECTIVE AND RESEARCH INTEREST

To become an independent researcher in the fields of molecular epidemiology and genetics, specifically to become an active investigator in the genetic basis of Cancer. Also to apply knowledge of cancer prevention and control practices to public health setting and to aid in the recruitment of minorities into cancer research and into clinical trials.

SKILLS

Data Analysis, SPSS, Microsoft Word/Excel/Power Point, PCR, Genotyping, Sequencing, DNA Extractions, Cytogenetics, FISH, Western/Southern Blotting, grant writing

EDUCATION

2000- Current **Georgetown University** Washington, DC

Pre-doctoral Candidate, Tumor Biology Training Program

- Finished 55 credits of graduate coursework
- Currently working of thesis entitled "DNA repair gene polymorphisms in Hereditary and Sporadic Breast Cancer"
- Attended Social Marketing class

July 2004-Aug. 2004 **NCI Summer Curriculum in Cancer Prevention** Rockville, MD

- The first part of the course, Principles and Practice of Cancer prevention and control, focused on concepts, methods, issues, and applications related to the field. The learning modules in the first part included: Ethics, law and policy; Diet; Biometric methodology; Prevention and Control of Organ Specific Tumors; Behavioral Science and Community Intervention; Health Disparities and Cancer Prevention in Diverse Populations; And Occupational Cancer.
- The second part, Molecular Prevention, focused on concepts, methods, issues, and applications of molecular biology in cancer prevention efforts and the genetics of cancer. Learned about basic laboratory methodology and theory of how molecular techniques are applied to molecular epidemiology, bionutrition, chemoprevention, biomarkers, and translational research.

May 2003- July 2004 **Johns Hopkins School of Public Health** Baltimore, MD

- Graduate of summer program in Epidemiology and Biostatistics Graduate of Summer program in Epidemiology and Biostatistics.
- Classes taken include Introduction to Epidemiology, Biostatistics and Genetic Epidemiology in Populations

May 29-30, 2003 **Georgetown University School of Nursing & Health Studies** Washington, DC

- This conference focused on the epidemiology of tobacco use, nicotine pharmacology, principles of addiction, drug interactions with smoking, and assisting patients with quitting.

Aug. 1996-May 2000 **Hampton University** Hampton, VA

- Graduated Cum Laude
- B.S., Molecular Biology

EXPERIENCE

July 2001-current Peter Shields, MD

Thesis Research Lab, Georgetown University

- Currently working of thesis entitled "DNA repair gene polymorphisms in Hereditary and Sporadic Breast Cancer"-*received Department of Defense pre-doctoral grant (90K) for this work*
- Using cytogenetic and genotyping techniques to establish genotype-phenotype association in breast cancer
- Performing DNA extractions with the M48 Bio-robot
- Performing DNA sequencing using Amersham's (General Electric) MegaBace Sequencer
- Performing real-time genotyping experiments using ABI 7900
- Used PCR/RFLP techniques in breast cancer case-control study of over 3000 samples in identify polymorphisms in several genes
- Analyzing data using statistical analysis tools such as excel and SPSS
- Maintained databases and biomarker core facilities

December 2004-April 2005 Gail Hansen

Intern, DC Department of Health: HIV and AIDS Administration-Surveillance

- Acted as a technical consultant by informing department of new techniques used in HIV detection methods and Methods in HIV and AIDS surveillance
- Lectured department staff about basic immunology, molecular biology methods to detect HIV, and methods to detect viral resistance
- Designed brochure for new "STARHS" (Serological Testing Algorhithm for Recent HIV Seroconverters) study to recruit patients, health care providers, and lab technicians into the study
- Designed STARHS information pamphlets for patients, health care providers and lab technicians

January- May 2001 Yan A. Su, PhD

Lab Rotation, Georgetown University

- Optimized protocol for utilization of human and mouse gene microarray filters
- Compared gene expression patterns of mouse xenografts from human cell lines

- Performed RNA extractions from human and mouse cell lines

July 2000-December 2000 Dorraya El-Ashry, PhD

Lab Rotation, Georgetown University

- Studied mechanism form tamoxifen resistance in ER- negative cell lines
- Maintained cell cultures for future experiments
- Performed western blots and luciferase assays to determine protein activity

May 1999- May 2000 William Kearns, PhD

Summer Assistantship in Oncology, NCI/Eastern Virginia Medical School and Center for Pediatric Research in conjunction with Minority Access to Research Careers, Hampton Univ.

- Used PCR/RFLP techniques to determine prevalence of unique polymorphisms in the GSTM1 and GSTT1 gene in patients with aplastic anemia and myelodysplatic syndromes
- Extracted DNA from patient bone marrow samples using Qiagen kit
- Used Fluorescence In Situ Hybridization techniques to determine ploidy in case-control study
- Analyzed data using statistical analysis tools
- Made media and reagents for future experiments

PROFESSIONAL MEMBERSHIPS

American Association for Cancer Research, Associate Member

Sigma Xi, Scientific Honor Society

AWARDS RECEIVED

Department of Defense Pre-doctoral Fellowship Award 2004-2007

AACR Minorities in Cancer Research Travel Fellowship – Nov. 2003, Feb. 2004, April 2005, April 2006

Georgetown University Pre-doctoral Fellowship- June 2000-June 2004

Minority Access to Research Careers Fellowship- 1999-2000

Lucille Packard Scholarship- Aug. 1997-2000

Alliance for Minority Participation in Science Fellowship- May 1997- May 2000

SCIENTIFIC MEETINGS AND CONFERENCES ATTENDED

American Association for Cancer Research Annual Meeting April 2004, 2005, 2006

American Public Health Association Annual Conference November 2004

American Association for Cancer Research- Radiation Biology and Cancer February 2004

American Association for Cancer Research- SNPs, Haplotypes, and Cancer November 2003

Lombardi Cancer Center Research Fair 2002-2004

Student Research Days, Georgetown. University 2002-2004

BIBLIOGRAPHY

In preparation:

Ricks-Santi, L. et al. **Mutagen sensitivity in EBV-immortalized cell lines correlated to fresh peripheral blood lymphocytes using the G2 chromatid break assay.**

Ricks-Santi, L. et al. **Breast Cancer DNA Repair genotypes and phenotypes**

DNA REPAIR GENE GENOTYPES AND ASSOCIATED PHENOTYPES IN BREAST CANCER

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Dissertation Advisor: Peter Shields, M.D.

ABSTRACT

Genetic susceptibilities for breast cancer can be elucidated by studying genotype-phenotype correlates. To study DNA repair capacity, the mutagen sensitivity assay, known to be associated with risk in familial and sporadic breast cancer, can be used to identify genotype-phenotype relationships. This experimental method, when used for women with familial breast cancer, e.g., *BRCA1* mutation carriers, may elucidate genotypes that might affect BRCA1 penetrance and also might affect sporadic breast cancer risk. This study investigated the association between specific *BRCA1* SNPs and haplotypes, *Rad51* SNPs, and deficient DNA repair. EBV-immortalized lymphocytes from 138 women were available for study and underwent the mutagen sensitivity assay in EBV-immortalized lymphoblasts. We validated the use of immortalized cells for this method for laboratory and day-to-day variation, and by comparing the results with fresh blood studies in a subset of women (n=33). Then, genotypes and haplotypes were determined for *BRCA1* and *RAD51,* and the associations with the mutagen sensitivity were assessed. Positive associations were then tested as predictors of breast cancer risk in a population-based case control study from New York (n= 1165 cases and 2170 controls). The coefficient of variation for the assay in EBV-immortalized lymphoblasts was 46.03%. The results from the immortalized lymphocytes were highly correlated to freshly blood lymphocyte cultures (ρ=0.92, p≤0.01), and absolute levels were quantitatively similar. Women with breast cancer had a higher MSA than women without breast cancer (OR=2.0, 95% CI: 0.90-4.6). An association was found for the *Rad51 135C* allele (rs1801320) and MBC, where OR=3.40 (95% CI: 1.20-9.90). There was an elevated OR when subjects were stratified by the *BRCA1* founder mutations (185delAG and 5382insC), although the numbers became small and the results were not statistically significant. There also was an increased risk for MBC with the *D693N* (rs4986850) allele (OR= 6.03 95%CI: 0.69-52.02), but this was not statistically significant (p=0.10). There was no association with for the *BRCA1 Q356R (*rs1799950*),* and *E1038G* (rs16941), but when stratified by 185delAG and 5382insC mutations, the BRCA1 *E1038G CT* genotype was associated with high MBPC in *185delAG* mutation carriers. For the CC and CT combined genotypes, the OR was also elevated (OR=8.0, 95% CI:0.6-106.9; p=0.14). Additionally, in *5382insC* mutation carriers ORs for the variant alleles for *D693N* and *Q356R* were 0.4 (0.01-8.1) and 0.3 (0.01-6.40), respectively, showing that having these variant alleles was associated with low MBPC, but results were not statistically significant (p=0.73 and 0.29, respectively). The *Rad51 135C* allele (rs1801320) was tested in a population-based case-control study of breast cancer, but no association was found. The OR was 0.83 (95% CI: 0.56-1.23) in premenopausal and 1.19 (95% CI: 0.93-1.51) for postmenopausal women. This study demonstrates that the use of EBV-immortalized lymphoblasts, as archived from previously conducted epidemiological studies, is methodologically valid for the assessment of cancer risk in epidemiological studies. This study provides data to justify a well-designed epidemiology study of the MSA as a predictive

phenotype in *BRCA1* mutation carriers and breast cancer risk. The results indicated that the *Rad51 135C* allele, and maybe the *D693N, Q356R,* and *E1038G* alleles, is modifying genotypes for the penetrance of *BRCA1* mutation carriers. The *RAD51* genotype, however, was not associated with sporadic breast cancer risk, and so might only be risk factor for high risk families.